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Review Article

Mechanisms for Chemical Reactions Involved in Lignin Biodegradation by *Phanerochaete chrysosporium*[*1]

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Keywords: fungal degradation of lignin, *Phanerochaete chrysosporium*, lignin substructure model compounds, aromatic ring cleavage, side chain cleavage

Contents

*1 This review artcle is the abstract of the Ph. D. thesis by the author (Kyoto University, 1987) entitled "Mechanisms for chemical reactions involved in lignin biodegradation by *Phanerochaete chrysosporium*".

*2 Research Section of Lignin Chemistry.

Introduction

The word "lignin" is derived from the Latin word "lignum" meaning wood. Lignin is one of the main constituents of xylem tissue, and its content is in the ranges of 24–33% in gymnosperms and 16–24% in angiosperms[1]. Hence, it has been stated that lignin is a second abundant renewable organic compound next to cellulose. Another important feature of lignin is that lignin is one of the most structurally complex biopolymers, having a variety of intermonomer linkages (lignin substructures) as well as different monomer structures. Model structures of lignin have been proposed by many investigators mainly based on the relative frequency of occurrence of each substructure (Fig. 1)[2].

Understanding of lignin biodegradation is important not only from the view-

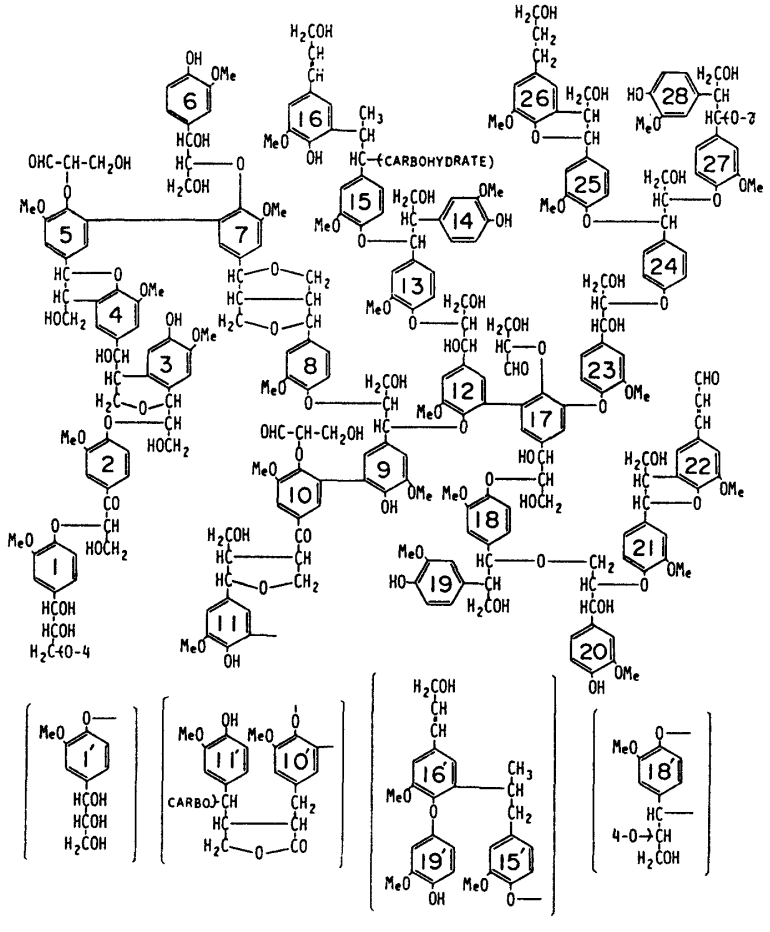

Fig. 1. Schematic formula for conifer lignin (Sakakibara, 1980)[2].

point of pure biochemistry and physiology but also for applied sciences. Due to abundance of lignin, lignin biodegradation is considered as one of the major biochemical processes occurring in the earth's carbon cycle. Unlike other biopolymers such as cellulose, polypeptides and nucleic acid, lignin is racemic and has no strict repeating units. Accordingly, "enzymes" mediating lignin biodegradation have been expected to be unique and less specific rather than those involved in usual highly specific metabolisms. As for application of lignin biodegradation, economical requirements due to the increase in oil price and shortage of fossil resources have accelerated researches on chemical and biochemical conversion of abundant lignocellulosic materials into more valuable chemicals and fuels.

According to the types of wood decay, wood-rotting fungi are classified into three categories; white-rot fungi, brown-rot fungi and soft-rot fungi. In addition, lignin-degrading bacteria and actinomycetes have been found. Among them major lignin degraders are white-rot fungi that are classified mainly as Basidiomycetes. Microbial lignin degradation has been studied by two complementary approaches: (i) fungal degradation of polymeric lignins (dehydrogenation polymer of coniferyl alcohol, milled wood lignin, or wood) and (ii) fungal degradation of lignin substructure model compounds. Chemical analyses of decayed lignin isolated from decayed wood have provided a general view of the chemistry of lignin biodegradation without the knowledge of enzymatic or mechanisms of specific degradative reactions of lignin. This is mainly because of the fact that lignin is very complex and heterogeneous polymer having a variety of intermonomer linkages (lignin substructures) (Fig. 1). Lignin substructure model oligomers having unequivocal structures are indispensable for elucidation of the specific chemical reactions involved and also for isolation of enzymes responsible for the reactions[3]. In the model studies, β-O-4 lignin substructure models (arylglycrol-β-aryl ethers, *e.g.* compounds (I) to (VI) in Fig. 2) have been used frequently, since β-O-4 substructure is the most prevalent intermonomer linkage in lignin (intermonomer linkages 1–2, 2–3, 8–9, 12–13, 17–18, 20–21, 23–24, and 24–27 in Fig. 1)[2].

Until the early 1980s when the author started this study, decayed lignins isolated from decayed wood were studied by many investigators, and the results were recently reviewed by Chen and Chang[4]. At least the three modes of degradative reactions were suggested to occur in the decomposition of lignin macromolecule by white-rot fungi: (i) Oxidative cleavage of side chains between α- and β-carbons (Cα-Cβ cleavage) leading to the formation of aromatic acids, (ii) cleavage of β-aryl ether bonds (β-O-4 bond cleavage) and modification of side chain structures, and (iii) degradation of aromatic nuclei through oxidative ring opening.

As for the knowledge of degradation of lignin substructure models by white-

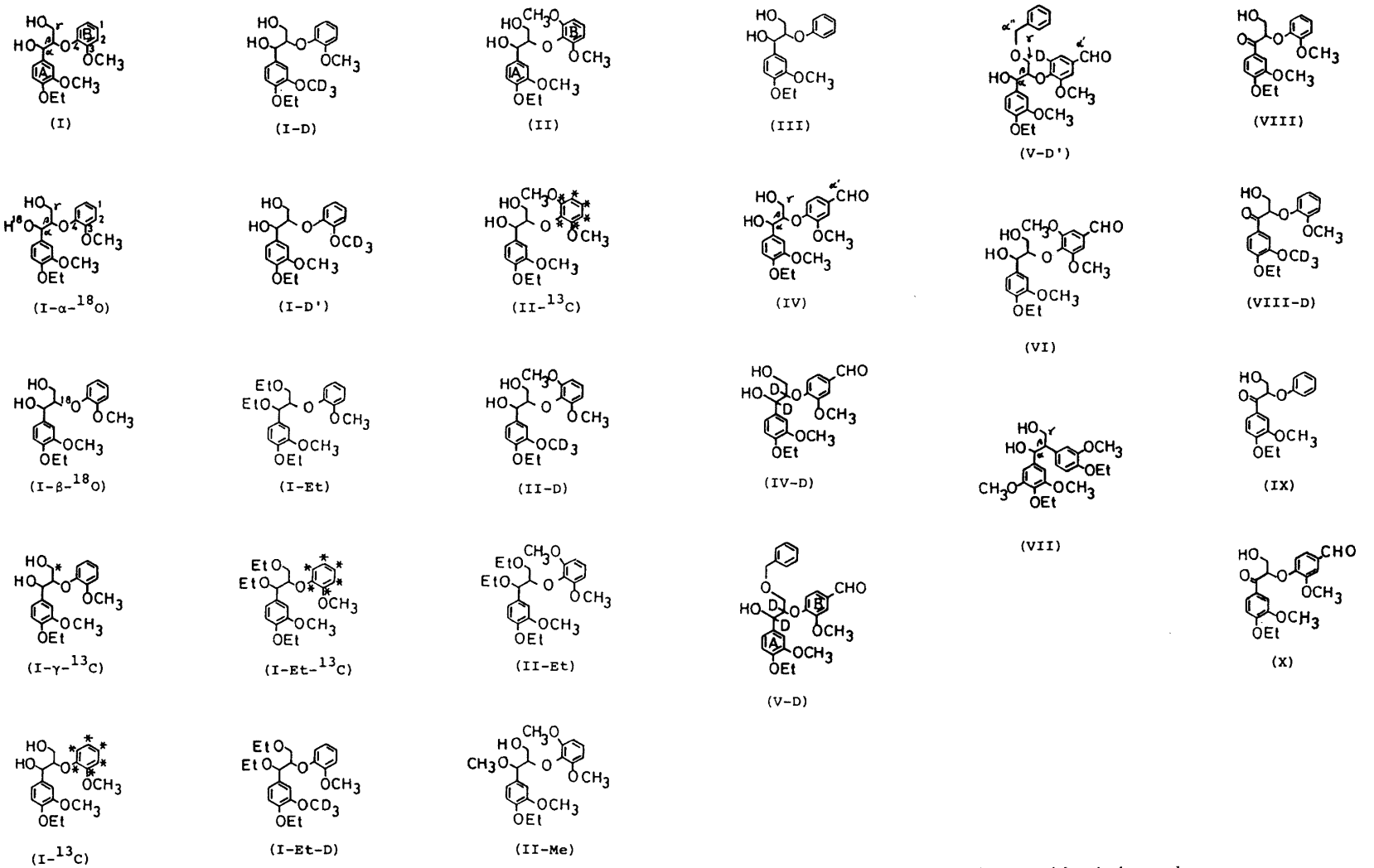

Fig. 2. Chemical structures of compounds. *: ^{13}C, D: ^{2}H, Et: CH_2CH_3. The system to denote side-chain carbon atoms (α, β and γ) is commonly used in lignin chemistry.

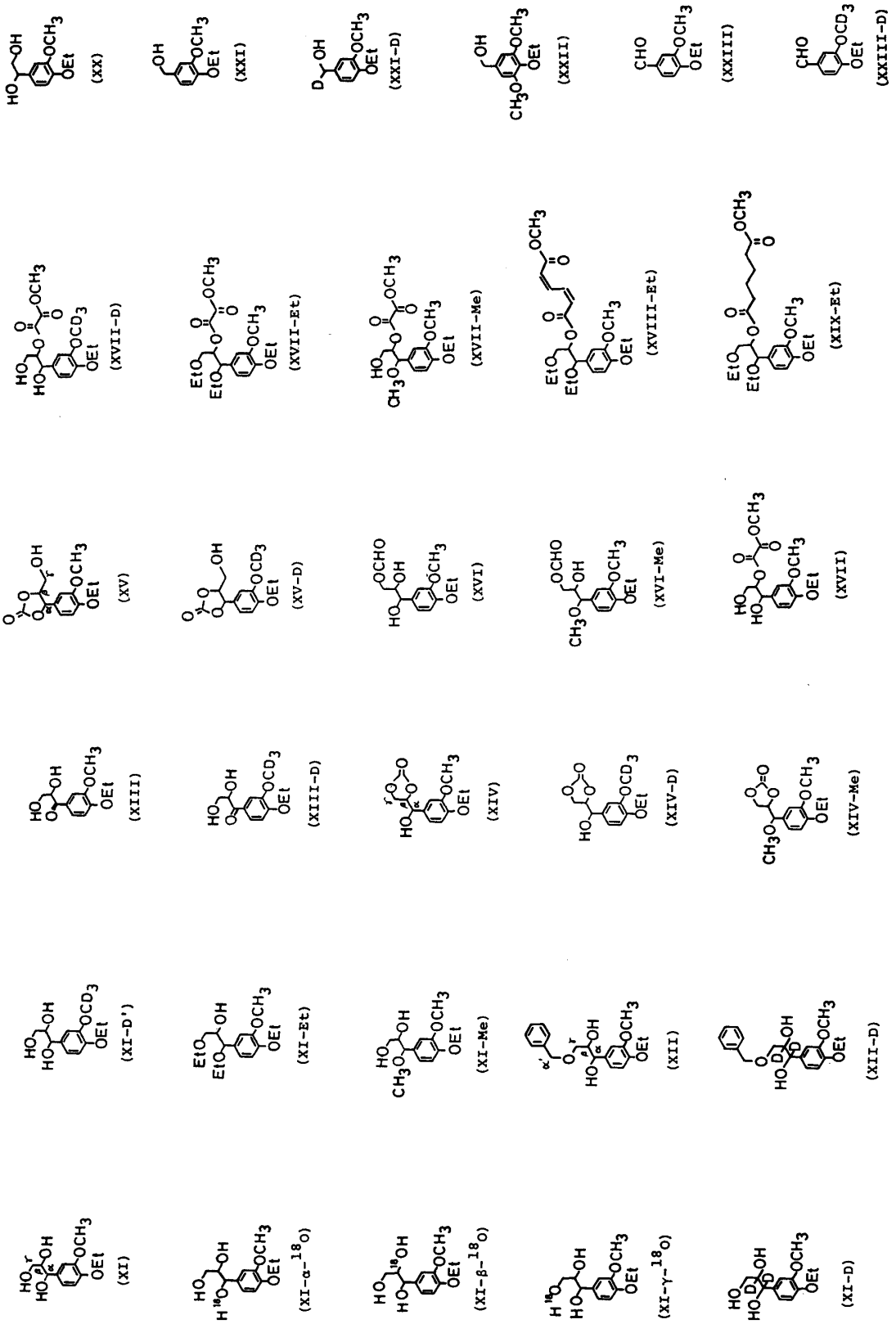

Fig. 2. (cont'd)

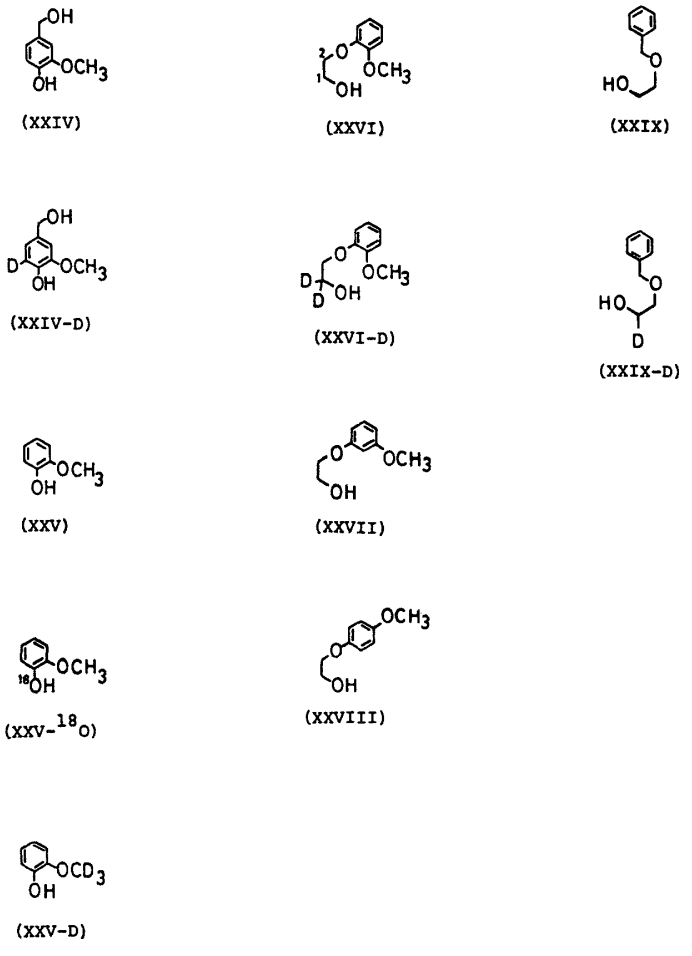

Fig. 2. (cont'd)

rot fungi in the early 1980s, some of the degradative reactions suggested by the studies of polymeric lignin degradation were identified, but their mechanisms remained ambiguous, and mixed results were obtained as follows. In the 1960s the first study of degradation of β-O-4 lignin substructure models by white-rot fungi was conducted by Russell *et al.*[5]. The study and a later paper by Kirk *et al.*[6] indicated that non-phenolic β-O-4 models were not degraded by white-rot fungi, *Coriolus versicolor* (=*Polyporus versicolor* or *Polystictus versicolor*) or *Stereum frustulatum*, whereas phenolic models were degraded. Kirk *et al.*[7] found Cα-oxidation and Cα-phenyl cleavage of phenolic β-O-4 lignin substructure models catalyzed by laccase, which is known to be the enzyme responsible for Bavendamm's reaction specific for white-rot fungi[8]. However, since the non-phenolic compound used by these two groups well represents non-phenolic β-O-4 lignin substructure, it should have been degraded by the cultures. Kirk *et al.* later considered that the cultural conditions, but not the model compounds used in the earlier investigation, were probably not suitable for lignin degradation[9]. They investigated to optimize culture conditions for lignin metabolism by white-rot

fungi, and established in 1978 the optimal culture parameters for white-rot basidiomycete, *Phanerochaete chrysosporium*: Nitrogen-limiting, high O_2 partial pressure, and stationary cultures are required for expression of the ligninolytic activity[10].

On the other hand, the cleavage of β-aryl ether bond of a non-phenolic β-O-4 lignin substructure model, 1-(3, 4-dimethoxyphenyl)-2-(2-methoxyphenoxy)-1, 3-propanediol, by white-rot fungi, *Fomes fomentarius* and *Poria subacida* was reported by Ishikawa *et al.*[11] and Fukuzumi *et al.*[12]. Based on chromatographic identification, they suggested formation of guaiacylglycerol [1-(4-hydroxy-3-methoxyphenyl)-1, 2, 3-propanetriol] and guaiacol as products of the cleavage of β-aryl ether (β-O-4) bond. Two mechanisms were postulated for the β-O-4 bond cleavage to give the arylglycerol: (i) hydrolysis of the β-O-4 ether bond[13] and (ii) initial hydroxylation at the Cβ position of the β-O-4 model, followed by decomposition of hemiketal formed, and reduction of aryldihydroxyacetone intermediate to give arylglycerol (Fig. 7 (1))[12]. Enoki *et al.*[14,15] confirmed, based on mass spectrometric identification, the formation of arylglycerol and guaiacol from a arylglycerol-β-guaiacyl ether, [1-(4-ethoxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)-1, 3-propanediol], in the optimized (ligninolytic) culture of *P. chrysosporium*. But no concrete evidence to support the two mechanisms proposed earlier[12,13] has yet been provided.

Cα-Cβ cleavage of lignin substructure models with the ligninolytic culture of *P. chrysosporium* was also demonstrated in the early 1980s. The non-phenolic β-O-4 dimer, 1-(4-ethoxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)-1, 3-propanediol, was incubated with the same fungus, and 4-ethoxy-3-methoxybenzyl alcohol and 2-guaiacoxyethanol were identified as Cα-Cβ cleavage products[14]. The fungal degradation of β-5 (phenylcoumaran) and β-1 (diarylpropane) lignin substructure models yielded similar Cα-Cβ cleavage products[16–19]. The Cα-Cβ cleavage products were firmly identified by comparison of their ^{1}H-NMR and/or mass spectra with those of synthesized authentic samples. However, mechanisms for the Cα-Cβ cleavage reactions were still ambiguous. After all, in the early 1980s, among the three types of degradation suggested by degradation studies of lignin polymer by white-rot fungi, only the Cα-Cβ cleavage and β-O-4 bond cleavage to give arylglycerol were demonstrated in the fungal degradation of lignin substructure models. Thus, next problems were to elucidate the mechanisms for the Cα-Cβ cleavage and the β-O-4 bond cleavage, to identify aromatic ring cleavage products, and to isolate enzymes involved.

First of all, the present author examined the mechanisms for the cleavage of Cα-Cβ and β-O-4 bonds by *P. chrysosporium* (Chapter 1). The fungus was used, because it was the only fungus, culture conditions of which had been optimized for lignin degradation[10]. The other, even more important, reaction suggested by the analyses of decayed lignin isolated from decayed wood is cleavage of aromatic rings.

In Chapter 2, first identification of aromatic ring cleavage products was described.

In Chapter 3, the enzyme responsible for the aromatic ring cleavage was described. Extracellular lignin peroxidase of *P. chrysosporium* was found for the first time to catalyze aromatic ring cleavage of β-O-4 lignin substructure model dimers. Based on the results of tracer experiments by the use of $^{18}O_2$, $H_2{}^{18}O$ and deuterated substrates, the mechanism for the ring cleavage was proposed.

Figure 2 shows chemical structures of compounds dealt with in the present investigation. The symbol "Ac" in the registry number of a compound represents acetate, (I-Ac): acetate of (I).

1. Cleavage in Propyl Side Chain by *Phanerochaete chrysosporium*

In the early 1980s, studies of degradation of lignin substructure models had shown Cα-Cβ cleavage and β-O-4 bond cleavage to give arylglycerol in the degradation of β-O-4 lignin substructure models by white-rot fungi, especially *Phanerochaete chrysosporium*[11–15]. However, mechanisms for the reactions were not elucidated. In this chapter, mechanisms for the arylglycerol formation and Cα-Cβ cleavage were investigated by the use of β-O-4 lignin substructure models and stable isotopes.

1. 1 Two Alternative Pathways of Degradation of β-O-4 Lignin Substructure Models

1. 1. 1 Introduction

In the present investigation, degradation of a lignin model trimer containing α-O-γ and β-O-4 substructures (intermonomer linkages 18–20–21 in Fig. 1), 3-benzyloxy-1-(4-ethoxy-3-methoxyphenyl)-2-(4-formyl-2-methoxy [6-^{2}H] phenoxy)-1-propanol (V-D′), by *Phanerochaete chrysosporium* was examined. This compound, arylglycerol-γ-benzyl-β-vanillin diether (V-D′), is a simplified trimer model for a junction substructure connecting two chains in lignin polymer, and has C_1 side chain on the B-nucleus (formyl group). The compound, therefore, represents β-O-4 lignin substructure more accurately than arylglycerol-β-guaiacyl ethers used by the earlier investigators, which have no alkyl side chains on the B-nuclei. A novel Cα-Cβ cleavage pathway as well as the β-O-4 bond cleavage to give arylglycerol were found[20,21].

1. 1. 2 Results

Culture conditions

Phanerochaete chrysosporium Burds. (ME-446) was used. Experimental cultures were grown at 39°C without agitation in a nitrogen-limiting, glucose, dilute mineral salts medium buffered with poly (acrylic acid) (pH 4.5, 0.01 M in carboxyl)[10,16,17].

Degradation of β-O-4 and α-O-γ lignin substructure trimer (V-D′)

86 mg of β-O-4 and α-O-γ trimer (V-D′) was added to 13 cultures (5- to 6-day old, 6.6 mg/20 ml culture). The cultures were incubated for 90 hr at 39°C without

agitation. Products extracted from the cultures were acetylated and separated twice by silica gel TLC. The fraction, Rf value of which was equal to that of acetate of vanillyl alcohol, was recovered from the gel. GC-MS analysis of the fraction showed the occurrence of acetate of [5-^{2}H] vanillyl alcohol (XXIV-D-Ac). The mass spectrum [MS m/z (%): 239(M^+, 3.8), 197(100), 155(58.1), 138(90.6), 137(41.5)] and the retention time were identical to those of authentic sample.

β-O-4 and α-O-γ trimer (V-D') (5 mg) was incubated under an atmosphere of $^{18}O_2$ for 113 hours. The culture extract (4.4 mg) was acetylated and separated into two fractions by silica gel TLC. Both the fractions were analyzed by GC-MS. Two compounds as well as [5-^{2}H] vanillyl alcohol (XXIV-D-Ac) [molecular ion region of the mass spectrum, m/z (%): 242(5.7), 241(7.2), 240(14.7), 239(100)] were identified by comparison of the mass spectra and retention times on GC with those of authentic samples. One was acetate of arylglycerol-γ-benzyl ether (XII-

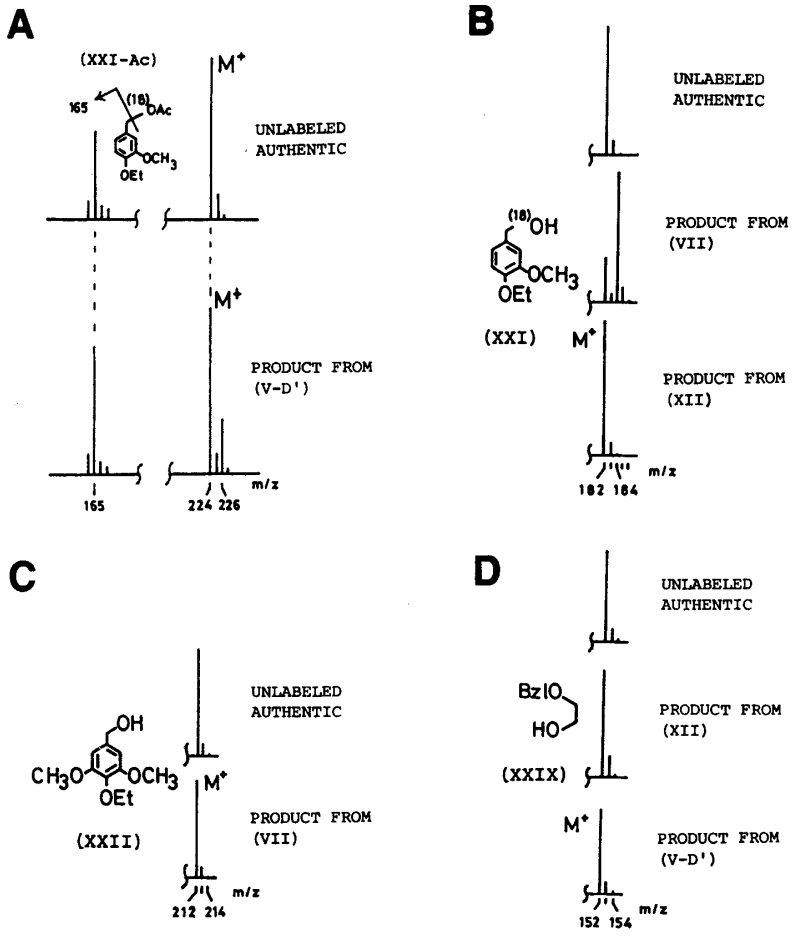

Fig. 3. Molecular ion regions of the mass spectra of products of degradation of β-O-4 and α-O-γ trimer (V-D'), β-1 dimer (VII) and arylglycerol-γ-benzyl ether (XII) by *Phanerochaete chrysosporium* under an atmosphere of $^{18}O_2$.

Ac) [MS m/z (%): 418(0.44), 417(1.6), 416(M^+, 5.6), 415(0.53), 356(2.7), 314(11.1), 223(28.2), 181(100), 180(6.2), 91(77.3)]. The other was acetate of 4-ethoxy-3-methoxybenzyl alcohol (XXI-Ac) [MS m/z (%): 226(10.4), 224(31.8), 198(1.4), 196(3.7), 184(2.4), 182(11.9), 165(24.7), 156(6.0), 155(6.0), 154(24.5), 137(100)]. The mass spectrum (Fig. 3A) showed 25% of (XXI-Ac) contained 1 atom of ^{18}O. MS also indicated that ^{18}O was incorporated into the benzyl position of (XXI) (Fig. 3A). On a repeated run, products were analyzed, without acetylation and TLC separation, by GC-MS. 27% of (XXI) contained 1 atom of ^{18}O, and a new compound, 2-benzyloxyethanol (XXIX), was identified by comparison of its mass spectrum [m/z (%): 154(0.24), 153(1.1), 152(9.5), 107(30.7), 92(27.0), 91(100)] with that of authentic sample. The mass spectra showed no incorporation of ^{18}O from $^{18}O_2$ into arylglycerol-γ-benzyl ether (XII-Ac) and 2-benzyloxyethanol (XXIX) (Fig. 3D). As for [5-^{2}H] vanillyl alcohol (XXIV-D-Ac), relative intensity of M^++2 ion (m/z 241) was a little higher than that of authentic sample or calculated value. But, noise level of the spectrum was relatively high (*e.g.* m/z 242, 5.7%), the M^++2 ion, therefore, could not be attributed to ^{18}O incorporation. Uninoculated control culture did not mediate these degradative reactions.

Degradation of arylglycerol-γ-benzyl ether (XII)

After incubation with 1.95 mg of (XII) for 27 hr under $^{18}O_2$, the culture was extracted (0.7 mg). GC-MS analysis of the extract revealed that neither Cα-derived product (XXI) nor Cβ-derived product (XXIX) contained ^{18}O (Fig. 3B and D). Uninoculated control culture did not mediate formation of the products.

Degradation of β-1 dimer (VII)

GC-MS analysis showed that the extract of the culture incubated with 2.85 mg of (VII) under $^{18}O_2$ for 24 hr contained Cα-derived product, 4-ethoxy-3-methoxybenzyl alcohol (XXI) [MS m/z (%): 184(100), 182(33.2), 156(46.3), 155(34.7), 154(22.6), 153(32.6), 151(13.7), 137(65.3), 125(75.8), 124(33.7), 123(35.8), 122(16.8), 93(92.6)], and Cα-derived product 3,5-dimethoxy-4-ethoxybenzyl alcohol (XXII) [MS m/z (%): 212 (M^+, 100), 184(82.6), 183(39.1), 167(34.2), 155(29.3), 153(13.0), 151(15.2), 141(14.1), 140(13.0), 137(15.2), 127(59.8), 123(50.0), 109(34.8), 97(14.1), 95(45.7), 93(18.5)]. Phenylglycol (XX), which was found to be a primary product in Cα-Cβ cleavage of β-1 substructure model[18,19], was isolated from the extract by silica gel TLC separation. The mass spectrum of phenylglycol (XX) was m/z (%), 214(M^+ for ^{18}O-(XX), 13.2), 212(M^+ for ^{16}O-(XX), 4.0), 194(25.2), 183(84.8), 181 (25.2), 165(37.1), 155(14.6), 153(23.2), 137(100), 125(38.4), 93(85.6). These products were identified by comparison of the mass spectra with those of unlabeled authentic samples. Mass spectra revealed that Cα-derived product 3,5-dimethoxy-4-ethoxybenzyl alcohol (XXII) did not contain ^{18}O (Fig. 3C), while 76% of phenylglycol

(XX) and 79% of 4-ethoxy-3-methoxybenzyl alcohol (XXI) (Fig. 3B) contained 1 atom each of ^{18}O in the benzyl position which corresponds to the Cβ position of β-1 type dimer (VII) (Fig. 5).

1. 1. 3 Discussion

Based on the metabolites identified and the isotopic experiments, Fig. 4 was proposed as the metabolic pathway for a β-O-4 lignin substructure model, β-O-4 and α-O-γ lignin substructure model trimer (V-D′), in a ligninolytic culture of *Phanerochaete chrysosporium*.

Earlier investigators proposed two pathways for degradation of β-O-4 lignin substructure models (arylglycerol-β-aryl ethers) by white-rot fungi. One is the pathway *via* arylglycerol. A phenol which had been etherified to the Cβ position of the β-O-4 models was supposed to be the counterpart compound for the arylglycerol: guaiacol for arylglycerol-β-guaiacyl ethers[12,15]. The other pathway is Cα-Cβ cleavage of β-O-4 substructure models[14]. These pathways were proposed based on the identification of the following products, arylglycerol, the phenol which had been etherified to the β-position of the substrate and substituted benzyl alcohol derived from the A-nucleus (see compound (I) or (II) in Fig. 2): *e.g.* 1-(4-ethoxy-3-methoxyphenyl)-1, 2, 3-propanetriol (XI), guaiacol (XXV) and 4-ethoxy-3-methoxybenzyl alcohol (XXI), respectively, in the degradation of 1-(4-ethoxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)-1, 3-propanediol by *P. chrysosporium*[14,15]. The corresponding products, arylglycerol-γ-benzyl ether (XII), (XXIV-D) and (XXI) were formed in the degradation of β-O-4 and α-O-γ type trimer (V-D′) by the fungus. It was thus established that β-O-4 bond adjacent to α-O-γ bond is cleaved to produce aryl-

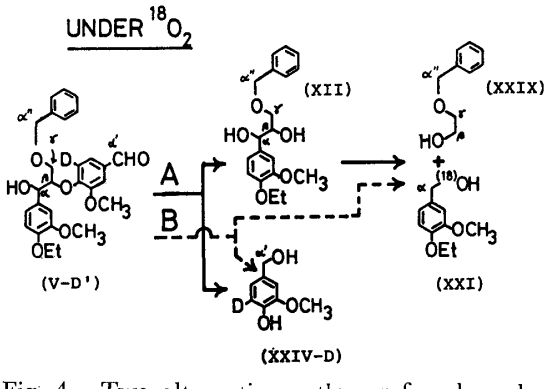

Fig. 4. Two alternative pathways for degradation of β-O-4 and α-O-γ trimer (V-D′) by *Phanerochaete chrysosporium*. Incorporation of ^{18}O from $^{18}O_2$ into (XXI) was *via* pathway B. α, α', α'', β and γ in the products represent that these carbon atoms were derived from Cα, Cα', Cα'', Cβ and Cγ of (V-D′), respectively.

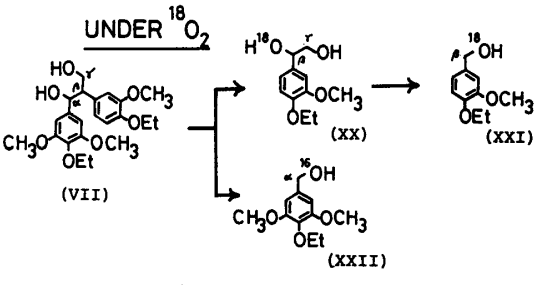

Fig. 5. Incorporation of ^{18}O from $^{18}O_2$ in degradation of β-1 dimer (VII) by *Phanerochaete chrysosporium*. α, β and γ in the products represent that these carbon atoms were derived from Cα, Cβ and Cγ of (VII), respectively.

glycerol as in β-O-4 bond carrying no adjacent α-O-γ bond, and that the β-O-4 bond was cleaved without cleavage of the α-O-γ bond. The possible *de novo* synthesis of vanillyl alcohol (XXIV) by the fungus which is known to synthesize the 4-*O*-methylated analog of (XXIV), veratryl alcohol[22,23], was negligible, if any, because vanillyl alcohol deuterated at the C_5 position (XXIV-D) which is not biosynthesized was found to be formed from arylglycerol-β-(deuterated vanillin) ether (V-D′) by the fungus. This result indicates β-O-4 bond cleavage (pathway A, Fig. 4) and Cα-Cβ cleavage of β-O-4 and α-O-γ trimer (V-D′) by the fungus, which is in accord with the previous results[14,15]. However, tracer experiments with $^{18}O_2$ showed that the mechanism for the Cα-Cβ cleavage proposed earlier[14] was not the case for the Cα-Cβ cleavage of (V-D′), and the mechanism proposed earlier was eliminated later (Section 1. 5).

In the degradation of β-O-4 and α-O-γ trimer (V-D′), ^{18}O was not incorporated from $^{18}O_2$ into arylglycerol-γ-benzyl ether (XII) but incorporated in the benzyl position of Cα-Cβ cleavage fragment, 4-ethoxy-3-methoxybenzyl alcohol (XXI). Two possible mechanisms were considered for the incorporation of ^{18}O into (XXI). One is a secondary incorporation of ^{18}O into (XXI) (*e.g.* ^{18}O incorporation during oxidation of (XXI) to the corresponding benzoic acid which may be reduced to give ^{18}O-(XXI)). This mechanism was eliminated, since ^{18}O was not incorporated from $^{18}O_2$ into (XXI) in the degradation of arylglycerol-γ-benzyl ether (XII) and a β-O-4 dimer, 1-(4-ethoxy-3-methoxy)-2-phenoxy-1, 3-propanediol (III) (data not shown) by the fungus. Accordingly, ^{18}O is thought to be incorporated into (XXI) through another mechanism, probably oxygenative Cα-Cβ cleavage. The "oxygenative" pathway does not involve arylglycerol-γ-benzyl ether (XII) as an intermediate, because ^{18}O was not incorporated into 4-ethoxy-3-methoxybenzyl alcohol (XXI) in the pathway (V-D′)→(XII)→(XXI). Furthermore, the mechanism for Cα-Cβ cleavage proposed earlier involves initial Cγ-oxidation followed by retroaldol condensation type Cα-Cβ cleavage to produce finally 4-ethoxy-3-methoxybenzyl alcohol (XXI), and is not "oxygenative"[14]. Thus, the "oxygenative" pathway found in the present experiment is a novel Cα-Cβ cleavage.

In conclusion, the following two alternative pathways for the degradation of the β-O-4 substructure models were evidenced: (a) a pathway *via* arylglycerol (XII) (pathway A in Fig. 4), (b) a pathway which does not involve arylglycerol as an intermediate, probably oxygenative Cα-Cβ cleavage of the β-O-4 substructure model (V-D′) to give (XXIX), (XXI) and (XXIV-D) (pathway B in Fig. 4). In the pathway A (Fig. 4) [5-^{2}H] vanillyl alcohol (XXIV-D) was proposed to be the counterpart for arylglycerol-γ-benzyl ether (XII). Further study, however, showed that it is not the case: The arylglycerol and the phenol which had been etherified to the

Cβ position of the substrate were found to be formed *via* different pathways (Section 1. 4).

In the Cα-Cβ cleavage of β-O-4 and α-O-γ type trimer (V-D′) or arylglycerol-γ-benzyl ether (XII) under $^{18}O_2$, 2-benzyloxyethanol (XXIX) (Cβ, γ-derived product) did not contain ^{18}O. However, this result does not mean no incorporation of $^{18}O_2$ into the Cβ position in the cleavage, because loss of ^{18}O *via* the exchange reaction between benzyloxyacetaldehyde, a putative precursor of (XXIX), and $H_2{}^{16}O$ could be possible. A mixture of [CH^{18}O] benzyloxyacetaldehyde (^{18}O: >59%) and [CH^{18}O]-4-ethoxy-3-methoxybenzaldehyde (^{18}O: >77%) was incubated in a 6-day-old culture for 30 min, and reduction products (XXIX) and (XXI) formed were examined for ^{18}O retention. Analyses of the MS data revealed that ^{18}O was completely lost in 2-benzyloxyethanol (XXIX) and that 45% of 4-ethoxy-3-methoxybenzyl alcohol (XXI) contained 1 atom of ^{18}O. Consequently, it is not certain whether or not $^{18}O_2$ is incorporated into the Cβ position in the Cα-Cβ cleavage of β-O-4 and α-O-γ trimer (V-D′).

In the Cα-Cβ cleavage of β-1 dimers by *P. chrysosporium*, Nakatsubo *et al.*[18] and Gold *et al.*[24] found that $^{18}O_2$ was incorporated into the Cβ position of the β-1 dimers, although incorporation of $^{18}O_2$ into the Cα position remained uncertain. In the present investigation, the β-1 dimer with different aromatic moieties (VII) was used as a substrate. A metabolite (XXII), the benzyl position of which corresponds to the Cα position of the substrate, did not contain ^{18}O (Fig. 5). This result indicates that $^{18}O_2$ was not incorporated into the Cα position in the Cα-Cβ cleavage of β-1 dimer (VII), because the possibility of the complete loss of ^{18}O *via* the exchange reaction between 3, 5-dimethoxy-4-ethoxybenzaldehyde, a primary product in the cleavage[25], and $H_2{}^{16}O$ was eliminated as mentioned above. Incorporation of $^{18}O_2$ into the benzyl position (Cβ position of the substrate) of phenylglycol (XX) and 4-ethoxy-3-methoxybenzyl alcohol (XXI) is in accord with the results reported previously[18,24]. Thus, it was established that $^{18}O_2$ was incorporated into Cα in opposite ways in the Cα-Cβ cleavage of the β-O-4 and β-1 substructure models.

Mechanisms for the Cα-Cβ cleavage of β-O-4 lignin substructure models by an extracellular enzyme of *Phanerochaete chrysosporium* is discussed in Section 1. 5.

1. 2 Mechanism for Arylglycerol Formation – Part 1 No Involvement of β-hydroxylation

1. 2. 1 Introduction

As described in Section 1. 1, two alternative pathways were evidenced in the degradation of a β-O-4 lignin substructure model by *Phanerochaete chrysosporium*: β-O-4 bond cleavage to give arylglycerol and Cα-Cβ cleavage[20,21]. In 1960s two mechanisms were proposed in order to explain the arylglycerol formation in cultures of white-

rot fungi. One is a pathway to involve initial hydroxylation at the β-position of the β-O-4 substructure[12], and the other is hydrolysis of the β-O-4 ether bond[13]. However, the mechanism for β-O-4 bond cleavage to give arylglycerol by white-rot fungi has not been elucidated completely. The present study using β-O-4 lignin substructure models deuterated at both Cα and Cβ positions showed that the β-hydroxylation mechanism is not valid for the β-O-4 bond cleavage producing arylglycerol by, at least, *P. chrysosporium*[20,26].

1. 2. 2 Results

Degradation of deuterated arylglycerol-β-vanillin ether (IV-D)

2.2 mg of [1, 2-$^2H_2$]-1-(4-ethoxy-3-methoxyphenyl)-2-(4-formyl-2-methoxyphenoxy)-1, 3-propanediol (IV-D) was incubated for 120 hr with *P. chrysosporium* and the degradation products were extracted and acetylated as described in the previous section. The acetylated product was separated by silica gel TLC to give a fraction, Rf value of which was approximately equal to that of triacetate of 1-(4-ethoxy-3-methoxyphenyl)-1, 2, 3-propanetriol (XI) (G-fraction). GC-MS analysis showed that the G-fraction contained triacetate of 1-(4-ethoxy-3-methoxyphenyl)-1, 2, 3-

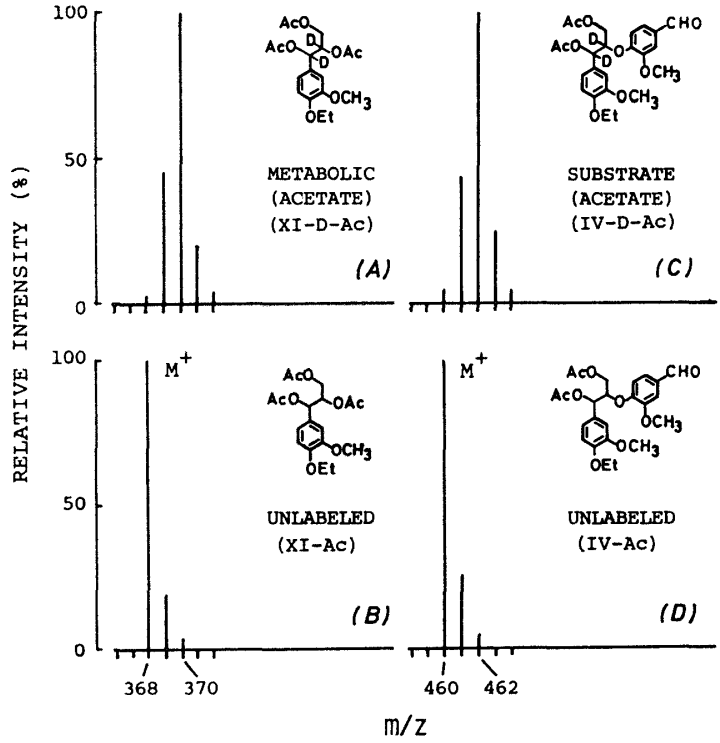

Fig. 6. Molecular ion region of the mass spectra of the substrate (IV-D-Ac) and the product of its degradation by *Phanerochaete chrysosporium* (XI-D-Ac). (A): Acetate of degradation product (XI-D-Ac). (C): Acetate of substrate (IV-D-Ac). (B) and (D): Unlabeled analogs of the product and the substrate, respectively.

propanetriol. The product gave a mass spectrum [m/z (%): 370(4.3), 369(2.1), 309(2.3), 308(1.0), 267(0.9), 252(0.8), 251(0.5), 250(0.3), 249(0.4), 224(6.9), 207(11.5), 182(100)] and a retention time identical to those of authentic samples. It is well known that a deuterated compound has a little smaller retention time than a non-deuterated analog in gas chromatography[27]. In order to avoid the isotope effect on the calculation of the content of deuterium atoms, ten mass spectra were taken successively at certain points of the peak in GC. Fig. 6 shows the relative abundance of molecular ion region of the mass spectra of the metabolic arylglycerol (the average value of the ten spectra) and that of the substrate, deuterated β-O-4 dimer, (IV-D) (the average value of eight spectra measured by direct inlet system). The mass spectra showed that 65.3% of the metabolic arylglycerol and 66.13% of the substrate (VI-D) contained two deuterium atoms excess to natural abundance at the α- and β-positions. The values were corrected for $M^+ + 1$ and $M^+ + 2$ of unlabeled and mono-deuterated analogs. Accordingly, 98.7% of hydrogen (deuterium) atoms at α- and β-positions of the arylglycerol were proved to be derived from those of the substrate (IV-D). Uninoculated control culture did not catalyze the formation of the arylglycerol (XI) from (IV).

Degradation of deuterated β-O-4 and α-O-γ trimer (V-D)

50 mg of [1, 2-$^2H_2$]-3-benzyloxy-1-(4-ethoxy-3-methoxyphenyl)-2-(4-formyl-2-methoxyphenoxy)-1-propanol (V-D) (5 mg/20 ml culture, ten cultures) was incubated with *P. chrysosporium* as above. The degradation products were extracted, and the extract was analyzed by GC-MS after silica gel TLC separation and acetylation, and the deuterium content of the product, arylglycerol-γ-benzyl ether (XII-D) (acetate) was evaluated. The product gave the mass spectrum, m/z (%): 419(M^++3, 0.87), 418(M^++2, 3.04), 417(M^++1, 1.74), 416(M^+, 0), 357(1.5), 315(7.6), 224 (22), 182(100), 181(8.6), 91(91), showing that the product was a mixture, double-deuterated at Cα and Cβ positions (M^++2, 418) and mono-deuterated (M^++1, 417), and that the ratio of the former/the latter was 6:4, which was equal to that of the substrate (V-D) (59 atom% ^2H at the Cβ position, 100 atom% ^2H at the Cα position). Uninoculated control culture did not catalyze the formation of the arylglycerol-γ-benzyl ether. Thus, the deuterium atoms of the substrate (V-D) was retained in the formation of the arylglycerol-γ-benzyl ether (XII-D) as in the formation of arylglycerol (XI-D) from deuterated β-O-4 dimer (IV-D).

1. 2. 3 Discussion

In 1960s, two reaction mechanisms were proposed for the cleavage of β-O-4 bond to produce the arylglycerol by white-rot fungi. One involves initial hydroxylation at the β-position of the β-O-4 substructure model (Fig. 7 (1))[12]. The other is hydrolysis of the β-O-4 bond[13]. The present investigation showed that the initial

β-hydroxylation was not involved in the degradation of β-O-4 lignin substructure models, at least, by *Phanerochaete chrysosporium*.

The present study evidenced that hydrogen (deuterium) atoms at Cα and Cβ of both the β-O-4 dimer (IV-D) and the β-O-4 and α-O-γ trimer (V-D) were retained at the Cα and Cβ positions of the arylglycerol (XI-D) and its γ-benzyl ether analog (XII-D), respectively, in the cleavage of the β-O-4 bond by *P. chrysosporium* (Fig. 8). Thus, it was established that any compound which lose deuterium atoms at Cα and/or Cβ of the β-O-4 type substrates with and without α-O-γ bond is not the intermediate compound to give the arylglycerol. Accordingly, as shown in Fig. 7, the following four intermediates were excluded in the arylglycerol formation accompanied by the β-O-4 bond cleavage: (1) β-carbonyl intermediate which could be produced by direct hydroxylation at β-position of the substrate proposed earlier[12] (Fig. 7 (1)), (2) α-carbonyl intermediate (Fig. 7 (2)), (3) an olefin with Cα-Cβ double bond by possible dehydration of the substrate (Fig. 7 (3)) and (4) α-hydroxycinnamyl alcohol derivative by possible elimination of the phenoxyl group attached to the β-position (Fig. 7 (4)).

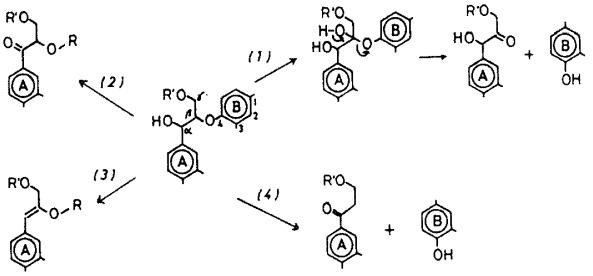

Fig. 7. Reactions which are not involved in the arylglycerol formation. In all the reactions, (1)–(4), hydrogen (deuterium) atoms at α- or β-position of the β-O-4 substructure are lost. R represents B-ring, hydrogen or an aliphatic group derived from B-ring. R′ represents hydrogen or α-O-γ bond.

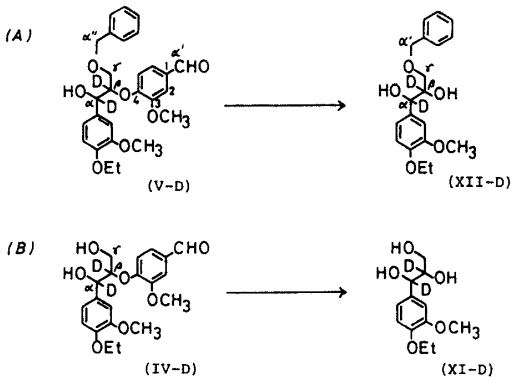

Fig. 8. Arylglycerol formation with retention of deuterium atoms at the α- and β-positions of the substrates in their degradation by *Phanerochaete chrysosporium*.

Cα-oxidation is one of prominent reactions found in the degradation of lignin by white-rot fungi[28,29]. α-Carbonyl formation in the degradation of a β-O-4 dimer by *P. chrysosporium* was reported[30]. But the present investigation proved that the α-carbonyl derivatives were not involved in the arylglycerol formation in the degradation of the β-O-4 substructure models with and without adjacent α-O-γ bond by *P. chrysosporium*, and that such α-carbonyl derivatives may be degraded *via* another pathway(s) which does not involve the arylglycerol.

1. 3 Mechanism for Arylglycerol Formation – Part 2 No Involvement of Direct Hydrolysis

1. 3. 1 Introduction

For the β-O-4 bond cleavage to produce arylglycerol by *Phanerochaete chrysosporium*,

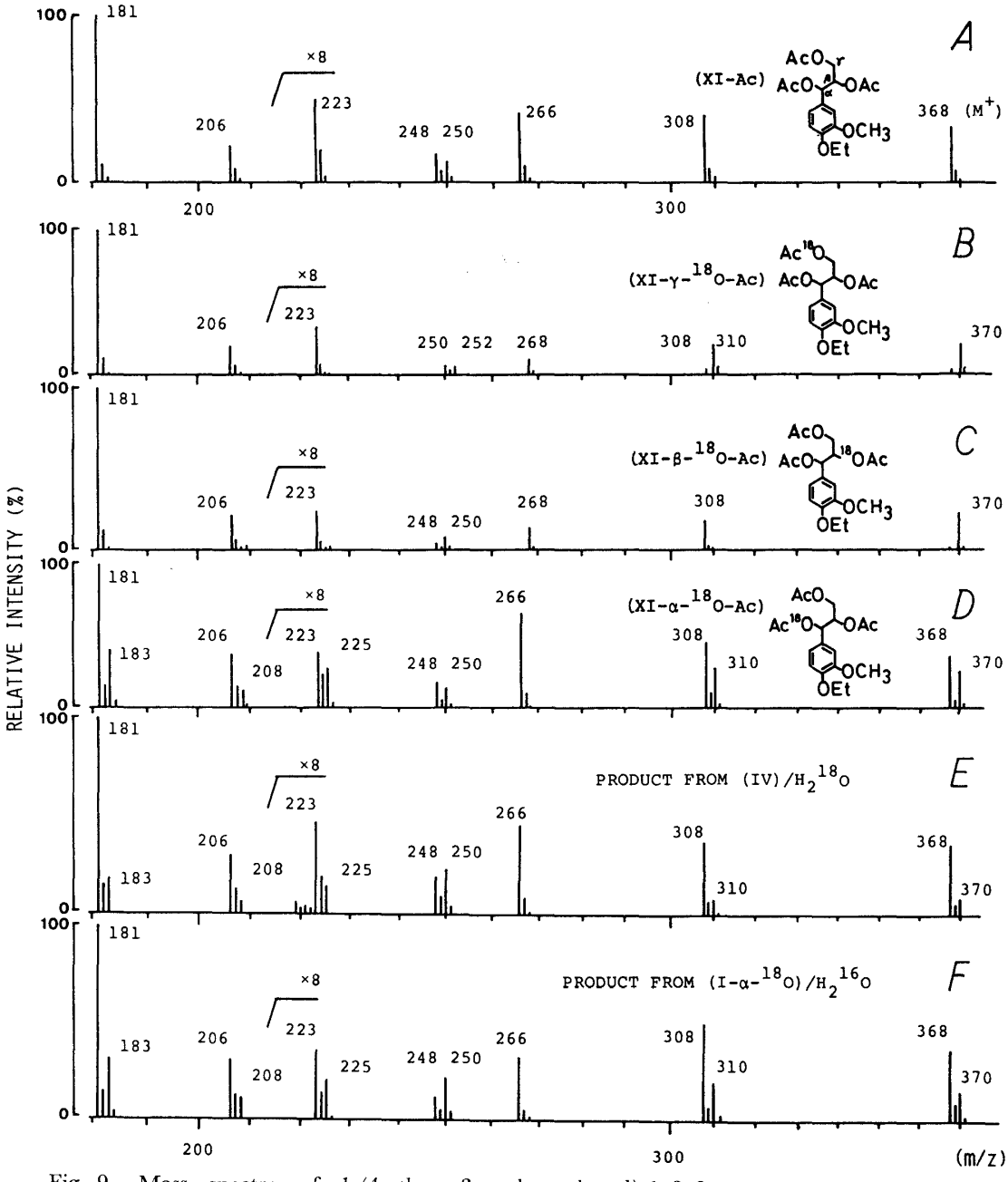

Fig. 9. Mass spectra of 1-(4-ethoxy-3-methoxyphenyl)-1, 2, 3-propanetriol (acetate). A, B, C and D: Synthesized compounds. E: Product of degradation of (IV) by *Phanerochaete chrysosporium* under $H_2{}^{18}O$. F: Product of degradation of (I-α-^{18}O) by the fungus (97 hr ext.). Based on the presence of the peaks at m/z 370, 310, 225, 208 and 183, and the absence of the peak at m/z 268 in E and F, it was concluded that both the products contained ^{18}O at Cα but not Cβ position.

no involvement of initial Cβ-hydroxylation was evidenced as described in the preceding section. This section discusses that the other mechanism for the arylglycerol formation suggested by earlier investigators, direct hydrolysis of the β-O-4 ether bond[13], was not correct in the arylglycerol formation at least by *P. chrysosporium*[31].

1. 3. 2 Results

Metabolism of arylglycerol-β-vanillin ether (IV) in a culture containing $H_2{}^{18}O$

After incubation of arylglycerol-β-vanillin ether (IV) for 96 hr in the culture of *P. chrysosporium* containing $H_2{}^{18}O$ (*ca.* 40%), metabolites were extracted, acetylated and analyzed by GC-MS as described in the precedent sections. The mass spectrum revealed that 17.0% of 1-(4-ethoxy-3-methoxyphenyl)-1, 2, 3-propanetriol (acetate) contained 1 atom of ^{18}O. The ^{18}O was found to be incorporated into the Cα position but not into the Cβ position by comparison of the mass spectra with those of ^{18}O-labeled authentic compounds, (XI-α-^{18}O-Ac), (XI-β-^{18}O-Ac) and (XI-γ-^{18}O-Ac) (Figs. 9 and 10A). Uninoculated control culture did not catalyze the conversion (IV)→(XI).

Metabolism of [1-^{18}O] arylglycerol-β-guaiacyl ether (I-α-^{18}O)

After incubation of ^{18}O-labeled arylglycerol-β-guaiacyl ether (I-α-^{18}O) (^{18}O: 48 atom % in the Cα position) in the isotopically unmodified cultures of *P. chrysosporium* for 73 hr and 97 hr, each culture was extracted, and the extracts were acetylated and analyzed by GC-MS as described in the precedent sections. GC-MS analysis showed that both extracts contained the acetate of 1-(4-methoxy-3-methoxyphenyl)-

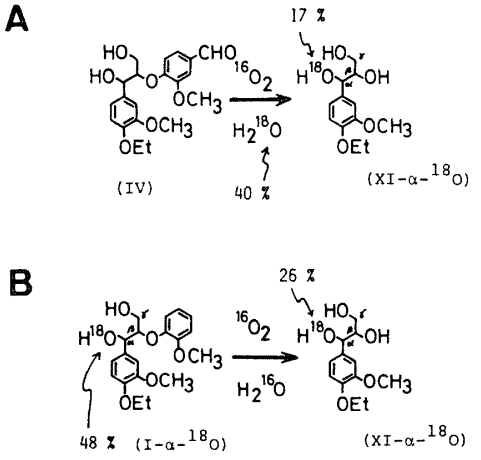

Fig. 10. No involvement of direct hydrolysis of β-O-4 bond in the degradation of β-O-4 lignin substructure models by *Phanerochaete chrysosporium*. ^{18}O was not incorporated from $H_2{}^{18}O$ into the Cβ position of 1-(4-ethoxy-3-methoxyphenyl)-1, 2, 3-propanetriol in the degradation of arylglycerol-β-vanillin ether (IV) by the fungus. About half of α-oxygen of the arylglycerol was derived from $H_2{}^{(18)}O$ and the other from the α-oxygen of the β-O-4 lignin substructure models.

1, 2, 3-propanetriol. It was further shown that 22.8% (73 hr ext.) and 26.1% (97 hr ext.) of the product (acetate) retained ^{18}O, and that the ^{18}O was found to be at the Cα position of its glycerol moiety by comparison of the mass spectra with those of ^{18}O-labeled synthesized authentic samples, (XI-α-^{18}O-Ac), (XI-β-^{18}O-Ac) and (XI-γ-^{18}O-Ac) (Figs. 9 and 10B). Uninoculated control culture did not catalyze the conversion (I)→(XI).

1. 3. 3 Discussion

Earlier investigators[11–13,15] proposed the participation of the following two mechanisms for the formation of arylglycerol: 1) Hydrolysis of the β-O-4 bond and 2) the mechanism involving initial hydroxylation at Cβ of the substructure. No involvement of the latter mechanism was evidenced as described in the precedent section. The present study of degradation of β-O-4 dimer (IV) by *P. chrysosporium* under $H_2{}^{18}O$ showed that the ^{18}O was not incorporated into the Cβ position of 1-(4-ethoxy-3-methoxyphenyl)-1, 2, 3-propanetriol (XI) (Fig. 10A). This result showed that the direct hydrolysis at the Cβ position of the β-O-4 bond was not involved in the cleavage of β-O-4 ether bond to form the arylglycerol (XI), although about half of the Cα oxygen of the arylglycerol (XI) was derived from H_2O ($H_2{}^{18}O$) and the other from the Cα oxygen of the substrate β-O-4 dimer (Fig. 10B). Thus, the two mechanisms for β-O-4 bond cleavage to produce the arylglycerol postulated by the earlier investigators were eliminated at least for *P. chrysosporium*.

As for the ^{18}O incorporation from $H_2{}^{18}O$ into the benzyl position of the glycerol, the following three results were obtained. i) The oxygen at the benzyl position (1- or α-position) of 1-(4-ethoxy-3-methoxyphenyl)-1, 2, 3-propanetriol (XI) was not exchanged with that of H_2O ($H_2{}^{18}O$): Incubation of synthesized [1-^{18}O] arylglycerol (XI-α-^{18}O) in an isotopically unmodified, uninoculated control culture at 39°C for 2 days did not lead to the loss of the ^{18}O. In addition, ^{18}O was not incorporated into the β-O-4 dimer (IV) recovered from the fungal culture after the incubation under $H_2{}^{18}O$. ii) It is plausible that the nucleophilic attack by the Cα oxygen on the β-O-4 substructure to Cα gives a Cα-Cβ epoxide which is subsequently attacked by H_2O to Cα giving the arylglycerol: A similar mechanism was proposed for the alkaline hydrolysis[32] and peroxyacetic acid oxidation[33] of the β-O-4 substructure. This pathway was, however, ruled out because of the absence of ^{18}O migration from the Cα position of β-O-4 dimer labeled with ^{18}O at the α-position (I-α-^{18}O) to the Cβ position of the arylglycerol. iii) The exchange of oxygen between the carbonyl group of the Cα carbonyl intermediate and $H_2{}^{18}O$ followed by reduction to give C$\alpha{}^{18}$OH was also excluded. The Cα carbonyl derivative was not an intermediate in the arylglycerol formation because in the degradation of β-O-4 lignin substructure models deuterated at both the Cα and Cβ positions by *P. chrysosporium*, the deuterium

atoms were retained at both the Cα and Cβ positions of the arylglycerols as described in the precedent section. For the β-ether cleavage, Enoki *et al.*[15] reported that the activity of the fungus giving the arylglycerol required neither the Cα nor Cγ hydroxyl function. Furthermore, the α-methyl or γ-benzyl ether (α-O-γ bond) of arylglycerol-β-vanillin ether was degraded to give the α-methyl or γ-benzyl ether of arylglycerol by the fungus without cleavage of the ether bond at Cα or Cγ as described in the precedent section (degradation of Cα-methyl ether: data not shown). However, the present investigation suggested that the ^{18}O was incorporated into the benzyl position of the arylglycerol from $H_2{}^{18}O$ at a stage closely associated with the β-ether cleavage and the arylglycerol formation, because simple exchange between the benzyl oxygen and that of $H_2{}^{18}O$, and the involvement of the Cα carbonyl intermediate were excluded. The ^{18}O incorporation mechanism will be discussed in Section 1. 5.

1. 4 Mechanism for Arylglycerol Formation – Part 3 O-C_4 Cleavage of β-O-4 Ether Bond

1. 4. 1 Introduction

In the precedent sections, mechanisms for the β-O-4 bond cleavage of β-O-4 lignin substructure models to give arylglycerol were investigated, and the mechanisms proposed by earlier investigators were found not to be correct in biodegradation at least by *Phanerochaete chrysosporium*. Next problem of the mechanism for arylglycerol formation was to elucidate whether the β-O-4 bond cleavage to give arylglycerol by the fungus proceeded *via* O-C_4 cleavage or Cβ-O cleavage, and to propose a novel mechanism for the arylglycerol formation. A β-O-4 lignin model dimer β-ethereal oxygen of which is labeled with ^{18}O was synthesized, and used as a substrate for fungal degradation, and the problem was successfully solved[34].

1. 4. 2 Results

[2-^{18}O]-1-(4-ethoxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)-1, 3-propanediol (I-β-^{18}O) was incubated for 92 hr with *P. chrysosporium* (4–5 mg/20 ml culture, 1–10 cultures) as in Section 1. 1. Metabolites were extracted with Et_2O from whole cultures acidified to pH 2 with 1 N HCl. The extract was acetylated, and separated by silica gel TLC to give two fractions, Rf values of which were approximately equal to those of triacetate of 1-(4-ethoxy-3-methoxyphenyl)-1, 2, 3-propanetriol (XI-Ac) (glycerol fraction) and acetate of guaiacol (XXV-Ac) (guaiacol fraction). GC-MS analysis showed that the glycerol fraction and the guaiacol fraction contained triacetate of 1-(4-ethoxy-3-methoxyphenyl)-1, 2, 3-propanetriol and acetate of guaiacol, respectively, as reported previously[15,31] (Section 1. 3). The excess ^{18}O of the arylglycerol was found to be located at Cβ position by comparison of the mass spectrum with those of authentic compounds, (XI-α-^{18}O-Ac), (XI-β-^{18}O-Ac) and (XI-γ-^{18}O-Ac)

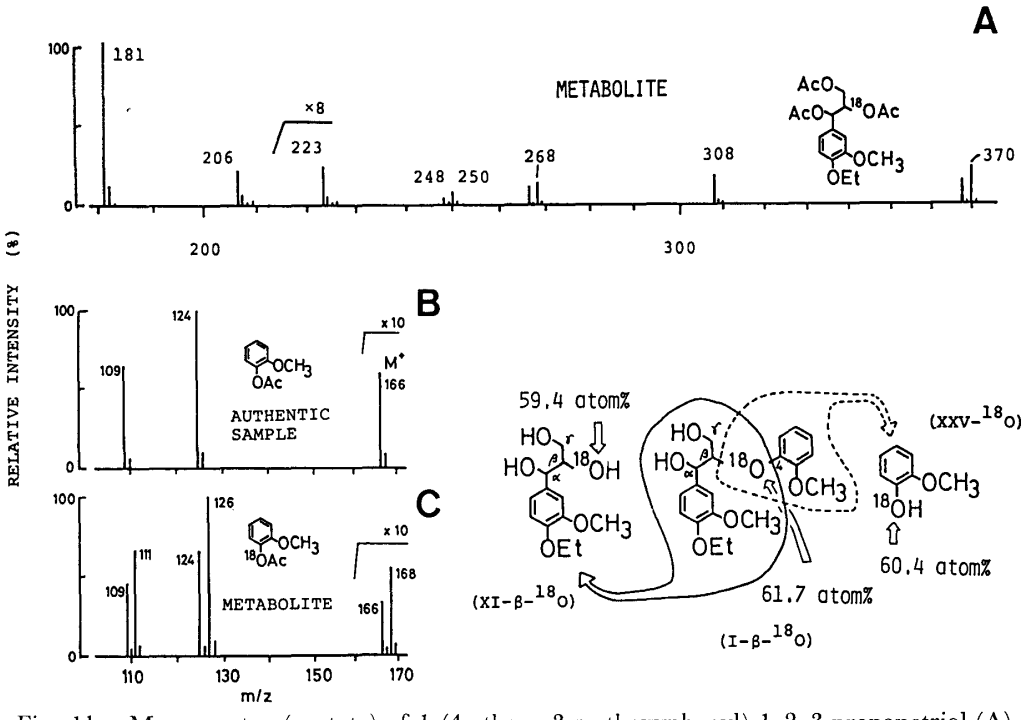

Fig. 11. Mass spectra (acetate) of 1-(4-ethoxy-3-methoxyphenyl)-1, 2, 3-propanetriol (A) and guaiacol (C), products of degradation of [2-^{18}O]arylglycerol-β-guaiacyl ether (I-β-^{18}O) by *Phanerochaete chrysosporium*. B: Authentic sample. β-Oxygen atom of the arylglycerol and phenolic oxygen atom of the guaiacol were derived quantitatively from β-ethereal oxygen of the substrate (I-β-^{18}O). Authentic samples for 1-(4-ethoxy-3-methoxyphenyl)-1, 2, 3-propanetriol are shown in Fig. 9.

(Figs. 9 and 11A). The mass spectrum of guaiacol (acetate) (Fig. 11C) was identical to that of synthesized (XXV-^{18}O-Ac). Relative intensity of the molecular ion region of the mass spectra of both metabolites are shown in Table 1. The ^{18}O content in Cβ of the arylglycerol and in the hydroxyl group of guaiacol was 59.4 and 60.4 atom% excess ^{18}O, respectively. Since the ^{18}O content in Cβ-O of (I-β-^{18}O)

Table 1. Relative intensity of molecular ion region of the mass spectra of acetate of arylglycerol (XI-Ac) and acetate of guaiacol (XXV-AC)

m/z	(XI-Ac) Relative intensity (%)		m/z	(XXV-Ac) Relative intensity (%)	
	Metabolite[a]	Unlabeled[b]		Metabolite[a]	Unlabeled[b]
366	0	0	164	0	0
368	66.1[c]	100	166	65.0[c]	100
370	100	4.9	168	100	1.3

a) Metabolite derived from (I-β-^{18}O). b) Unlabeled authentic sample. c) Average value in triplicate experiments [(XI-Ac): 63.9, 66.9 and 67.4; (XXV-Ac): 60.3, 66.5 and 68.2]. Each value is the average value from 6–19 different spectra in the three independent runs.

was 61.7 atom% excess ^{18}O, the result indicated that 96% of oxygen at Cβ of the arylglycerol and 98% of the phenolic oxygen of guaiacol were derived from Cβ-oxygen of (I-β-^{18}O), respectively. Acetate of [OC^2H_3] guaiacol (XXV-D-Ac) was synthesized and used as an authentic compound in TLC separation and GC-MS analysis. Even if guaiacol as metabolite would be contaminated for some unexpected reason with the authentic sample, calculation of the ^{18}O content of metabolic guaiacol is not affected, since (XXV-D-Ac) have no peaks from m/z 166 to 168 in the mass spectrum. Uninoculated control culture did not catalyze the conversion (I)→(XI). Selected ion monitoring suggested the formation of trace amount of guaiacol from (I) in the uninoculated culture (30% of that in the intact cells, data not shown).

1. 4. 3 Discussion

As described in Section 1. 1, two alternative pathways were proposed for degradation of the β-O-4 substructure model compound by *Phanerochaete chrysosporium* based on the results of ^{18}O incorporation from $^{18}O_2$. One is a pathway *via* the arylglycerol (Fig. 12, pathway A) and the other is a pathway which does not involve the arylglycerol, probably oxygenative Cα-Cβ cleavage, (Fig. 12, pathway B). The present result showed that guaiacol was not a counterpart compound for the arylglycerol but formed *via* a different pathway(s) in the cleavage of the β-O-4 bond by the fungus, since 98% of the phenolic oxygen of guaiacol and 96% of the oxygen at Cβ of 1-(4-ethoxy-3-methoxyphenyl)-1, 2, 3-propanetriol were derived from ethereal oxygen of β-O-4 bond of (I-β-^{18}O) in the β-ether cleavage by the fungus. If the two metabolites were counterpart compounds for each other, the ethereal oxygen of the β-O-4 bond would be shared with the two metabolites.

1-(4-Ethoxy-3-methoxyphenyl)-1, 2, 3-propanetriol (XI) was formed *via* O-C_4 bond cleavage which probably resulted from an initial attack on the aromatic ring of the Cβ phenoxyl group of arylglycerol-β-guaiacyl ether (I), although a counterpart compound for the arylglycerol (XI) is not yet known (Fig. 12, pathway A). Two

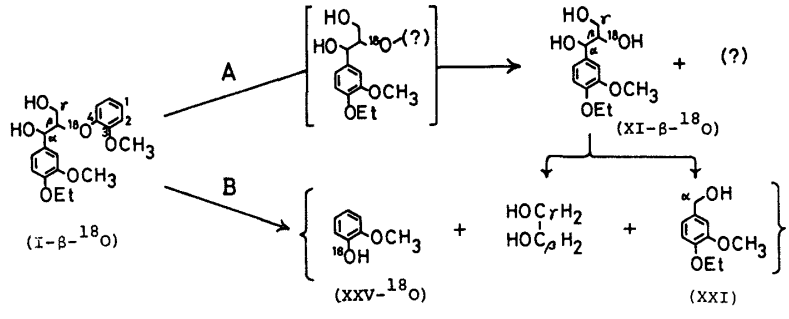

Fig. 12. Degradation pathways for arylglycerol-β-guaiacyl ether. Arylglycerol (XI-β-^{18}O) was formed *via* O-C_4 cleavage of β-O-4 bond, while guaiacol (XXV-^{18}O) was formed *via* Cβ-O cleavage of β-O-4 bond.

possible mechanisms may be deduced for the cleavage of the β-O-4 bond to give the arylglycerol. One is initial ring cleavage of phenoxyl group at Cβ. Chen *et al.*[35] suggested the cleavage of the aromatic ring of the Cβ phenoxyl group of lignin macromolecules before β-ether cleavage by the fungus. However, another mechanism which gives rise to a modified phenol differing from guaiacol or a quinone, as the counterpart compound for the arylglycerol (XI), may also occur in β-O-4 bond cleavage.

Tien and Kirk[36] and Glenn *et al.*[37] found an enzyme which catalyzes Cα-Cβ cleavage of lignin and lignin substructure models. Tien and Kirk[38] showed that the enzyme liberates the phenol etherified at the Cβ position of a β-O-4 lignin substructure model, and proposed a mechanism similar to pathway B in Fig. 12 for the Cα-Cβ cleavage to liberate the phenol by the enzyme. The mechanism for the Cα-Cβ cleavage by the enzyme will be discussed later (Section 1. 5).

1. 5 Cα-Cβ Cleavage with Rearrangement of the β-aryl Group

1. 5. 1 Introduction

In the previous sections, the mechanism for β-O-4 bond cleavage to give arylglycerol was mainly discussed. Another degradation product, 2-guaiacoxyethanol (XXVI), was first identified by Enoki *et al.*[14] as a product of Cα-Cβ cleavage of β-O-4 lignin substructure model dimer, 1-(4-ethoxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)-1, 3-propanediol (I), by *Phanerochaete chrysosporium*. In the present section, mechanism for formation of 2-guaiacoxyethanol (XXVI) is discussed. The mechanism was completely different from that proposed by earlier investigators[14], and was finally found to be closely related to that for the arylglycerol formation[39].

1. 5. 2 Results

After incubation of arylglycerol-β-guaiacyl ether (I) with *P. chrysosporium* for 93 hr as described in the precedent sections, acetate of 2-guaiacoxyethanol (XXVI-Ac) was isolated from the acetate of the culture extracts and identified by comparison of the mass and ^{1}H-NMR spectra with those of synthesized authentic samples, acetate of 2-guaiacoxyethanol (XXVI-Ac), acetate of 2-(*m*-methoxyphenoxy) ethanol (XXVII-Ac) and acetate of 2-(*p*-methoxyphenoxy) ethanol (XXVIII-Ac) (Fig. 13). When 2 mg of arylglycerol-β-guaiacyl ether (I) was added to 20 ml culture, the yield of 2-guaiacoxyethanol (XXVI) was 30 μg, 3% [(mol product formed/mol initial substrate) × 100] based on stable isotope dilution procedure using (XXVI-D) as an internal standard. Uninoculated control culture did not catalyze the conversion from (I) to (XXVI). When [2-^{18}O] arylglycerol-β-guaiacyl ether (I-β-^{18}O) (61.7 atom % excess ^{18}O in the β-position) was incubated with the fungus, GC-MS analysis of the acetate of the metabolites showed that ^{18}O was almost lost in the formation of 2-guaiacoxyethanol [MS, m/z (%): 210(5.5), 124(14.3), 109(21.7), 87(100)] (Table 2). In the incubation of [3-^{13}C] arylglycerol-β-guaiacyl ether (I-

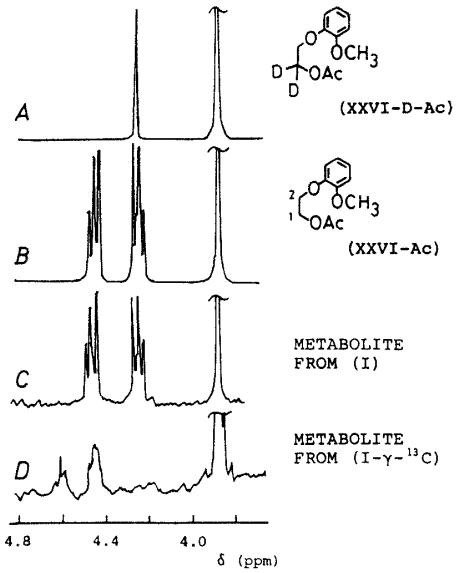

Fig. 13. A part of the ^{1}H-NMR spectra of 2-guaiacoxyethanol (acetate). (A) and (B): Synthesized compounds, (C) and (D): degradation product from (I) and (I-γ-^{13}C), respectively. In the spectrum B, peaks at 3.86, 4.23 and 4.44 ppm were assigned to methoxyl group, protons at the 2-position and at the 1-position, respectively, since in A, the peak at 4.44 ppm disappeared. Spectra B and C are identical, while the chemical shifts of the peaks of methoxyl groups of the *m*-isomer (XXVII-Ac) and *p*-isomer (XXVIII-Ac) were 3.79 and 3.77 ppm, respectively. Decoupling measurement showed that in spectrum D, the peak at 4.23 ppm is split into 4.60 and 3.86 ppm by coupling with ^{13}C ($J=148$ Hz). The peak at 4.44 ppm became a multiplet by coupling with ^{13}C ($J<10$ Hz). This result showed that the product from (I-γ-^{13}C) was labeled with ^{13}C at the 2-position, since $^{1}J_{C-H}$ and $^{2}J_{C-H}$ are usually >100 and <10 Hz, respectively.

Table 2. Relative intensity of molecular ion region of the mass spectra of the acetates of 2-guaiacoxyethanol.

m/z	Relative intensity (%)[d]		
	Metabolite 1[a]	Metabolite 2[b]	Unlabeled[c]
309	0	0	0
310	100	4.5	100
311	13.7	100	13.5
312	5.6	13.9	2.6

a) Metabolite from (I-β-^{18}O). b) Metabolite from (I-γ-^{13}C). c) Unlabeled authentic compound. d) Background correction was not made.

γ-^{13}C) as described above, MS analysis showed that the 2-guaiacoxyethanol formed retained the ^{13}C atom (Table 2), and the ^{13}C atom was found to be at 2-position of the 2-guaiacoxyethanol by ^{1}H-NMR analysis (Fig. 13).

1. 5. 3 Discussion

The possible formation mechanism for 2-guaiacoxyethanol (XXVI) from β-O-4 dimer (I) by *P. chrysosporium* was proposed as shown in Fig. 14. First, the guaiacyl group was rearranged to γ-position to give (c), which was subsequently cleaved between Cα and Cβ to give 4-ethoxy-3-methoxybenzaldehyde (XXIII) and 2-guaiacoxyacetaldehyde (e). Both (XXIII) and (e) were reduced to 4-ethoxy-3-methoxybenzyl alcohol (XXI) and 2-guaiacoxyethanol (XXVI). Reduction of (XXIII) and 2-benzyloxyacetaldehyde to (XXI) and 2-benzyloxyethanol (XXIX), respectively, by the fungus was described in Section 1. 1. This mechanism is entirely distinct from that proposed for the formation of (XXVI) by earlier investigators[14].

For the rearrangement of the guaiacyl group, two possible mechanisms are conceivable. One is activation of the guaiacyl aromatic ring (B-ring, Fig. 14) and the other is that of the γ-hydroxyl group of β-O-4 dimer (I). At present, the latter is not substantiated by any experimental results, while the former, activation of the aromatic ring, was recently reported by Kersten *et al.*[40]. They showed, based on ESR analysis, that *P. chrysosporium* ligninase (lignin peroxidase) produced cation radicals from methoxylated benzenes. A possible mechanism for the rearrangement of the guaiacyl group is as follows (Fig. 14). The guaiacyl (B) nucleus at the β-position of arylglycerol-β-guaiacyl ether (I) was oxidized to cation radical (a) (Fig. 14) by the ligninase (lignin peroxidase). The cation radical (a) was attacked by γ-hydroxyl group of the cation radical, followed by leaving of β-oxygen to give (c) (Fig. 14). The radical (c) was degraded directly (broken line) or *via* (d) to (XXIII) and (e). Recently, similar rearrangement catalyzed by the enzyme was reported[41].

Previous investigation (Section 1. 1) showed the Cα-Cβ cleavage of γ-benzyl

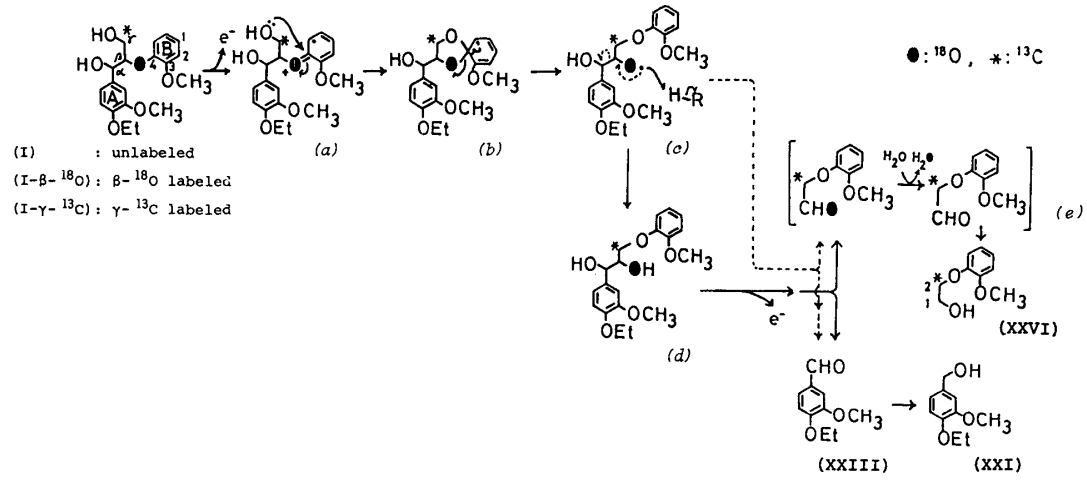

Fig. 14. A proposed mechanism for the formation of 2-guaiacoxyethanol (XXVI) from arylglycerol-β-guaiacyl ether (I) by *Phanerochaete chrysosporium*. (*a*)–(*e*): Assumed compounds.

ether analog of intermediate (d) (Fig. 14), arylglycerol-γ-benzyl ether (XII), by the fungus to give 4-ethoxy-3-methoxybenzyl alcohol (XXI) and 2-benzyloxyethanol (XXVI)[20,21] (Section 1. 1). Intradiol cleavage of phenylglycol structures to give benzaldehydes by lignin peroxidase was also reported[38,42]. Disappearance of ^{18}O in 2-guaiacoxyethanol (XXVI) in the degradation of [2-^{18}O] arylglycerol-β-guaiacyl ether (I-β-^{18}O) is explained by exchange between carbonyl oxygen of 2-guaiacoxyacetaldehyde and that of H_2O. Similar exchange between [$CH^{18}O$] benzyloxyacetaldehyde and H_2O in the same culture condition was reported in Section 1. 1[21]. Since preliminary experiment showed that ratio of yield, (XXVI)/(XXI), was about 0.4, the Cα-Cβ cleavage proposed here, which is distinct from the mechanism proposed by Tien and Kirk[38], may be important among Cα-Cβ cleavage pathways to give 4-ethoxy-3-methoxybenzyl alcohol (XXI).

In the following part of this section, the mechanisms for β-O-4 bond cleavage and Cα-Cβ cleavage of β-O-4 lignin models are discussed as the summary of Chapter 1. While the author was doing the experiments described in the Chapter 1, an extracellular enzyme of *P. chrysosporium* was isolated[36,37]. The enzyme, lignin peroxidase, was found to catalyze the formation of the arylglycerol (XI) from β-O-4 lignin substructure models, such as (I) and (II)[37,42–47] (Section 3. 1), and the Cα-Cβ cleavage of β-O-4 lignin substructure models[36–38,42–46] (Section 3. 1). Lignin peroxidase oxidizes substituted aromatic compounds including a β-O-4 lignin model dimer to aryl cation radicals[40,45]. The formation mechanisms of the arylglycerol (XI) as well as 2-guaiacoxyethanol (XXVI) are explained on the basis of initial single-electron oxidation of B-nucleus of the β-O-4 lignin models by lignin peroxidase, as shown in Fig. 15A. The mechanisms are in good accordance with the results by using intact cells of *P. chrysosporium* and stable isotopes described in Chapter 1: (i) Deuterium atoms at the α- and β-positions of the β-O-4 lignin models were retained in the arylglycerol formation (Section 1. 2, Figs. 8 and 15A), (ii) $H_2{}^{18}O$ was not incorporated into the Cβ position of the arylglycerol (Section 1. 3, Figs. 10A and 15A), (iii) Cβ-oxygen of the arylglycerol was quantitatively derived from the β-O-4 ethereal oxygen of the β-O-4 dimer (I) (Section 1. 4, Figs. 12 and 15A). ^{18}O incorporation from $H_2{}^{18}O$ into the Cα position of the arylglycerol (about half of the Cα-oxygen of the arylglycerol (XI) was derived from $H_2{}^{(18)}O$, and the other from the Cα-oxygen of the substrate (I-α-^{18}O), Section 1. 3, Fig. 10) was explained by the nucleophilic attack by Cα-hydroxyl function (pathway (a)→(d)→(e)→(XI), Fig. 15A). As described in Chapter 3, lignin peroxidase catalyzes aromatic ring cleavage of β-O-4 lignin models. Aromatic ring cleavage product (XIV) (Fig. 15A) is also a precursor of (XI), which is described in Section 2. 1. No experiments were conducted to confirm the retention of deuterium and β-oxygen atoms in the formation of (XIV)

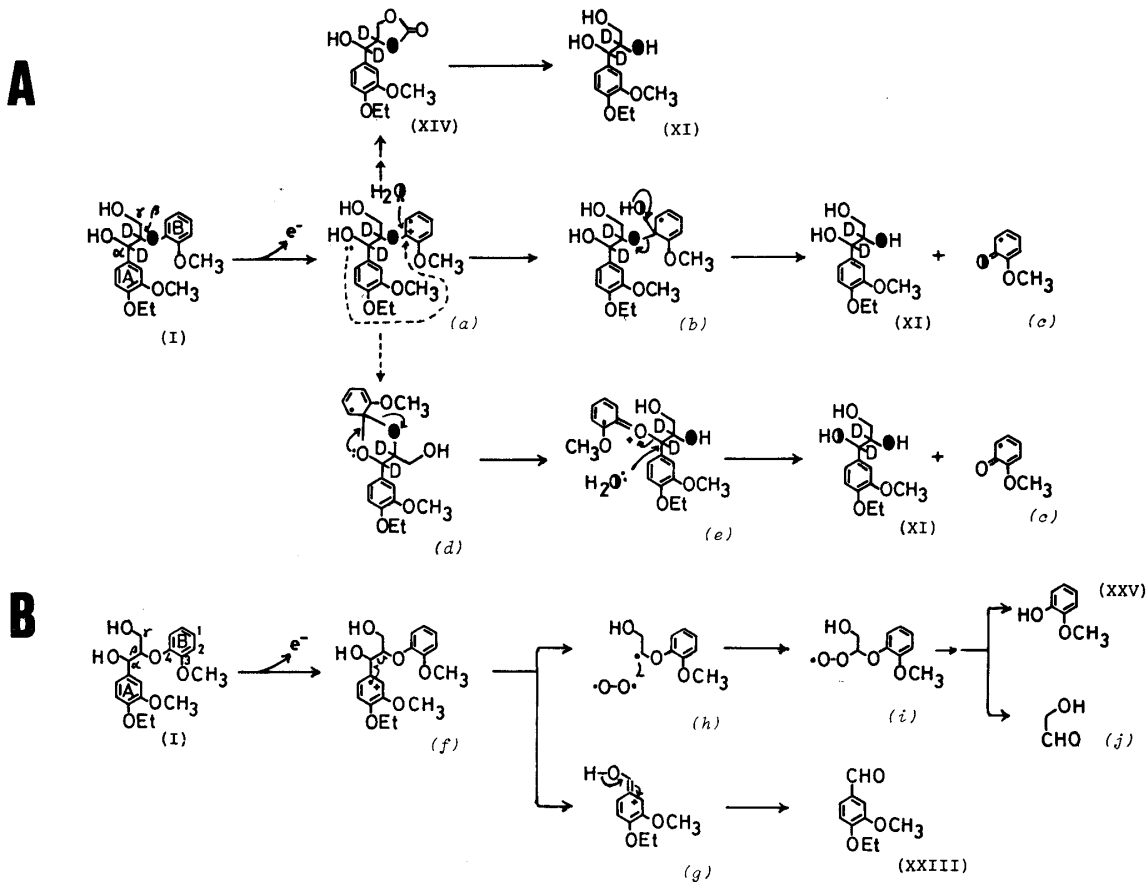

Fig. 15. Proposed mechanisms for the β-O-4 bond cleavage to give 1-(4-ethoxy-3-methoxyphenyl)-1, 2, 3-propanetriol (XI), (A), and Cα-Cβ cleavage (B) of arylglycerol β-guaiacyl ether (I) by *Phanerochaete chrysosporium*. ●: ^{18}O of, or derived from, β-ethereal oxygen of (I); ◑: ^{18}O of, or derived from, $H_2{}^{18}O$; D: ^{2}H. In this figure, (I) and (XI) represent 1-(4-ethoxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)-1, 3-propanediol and its labeled analogs, and 1-(4-ethoxy-3-methoxyphenyl)-1, 2, 3-propanetriol and its labeled analogs, respectively. (*a*)–(*j*): Assumed compounds. The pathway (I)→(XIV)→(XI) will be described in Section 2.1.

from (I), and (XI) from (XIV). Recently, the same results as (i), (ii) and (iii) were obtained by the use of lignin peroxidase of *P. chrysosporium*[43,44]. The mechanisms similar to the pathways (I)→(a)→(b)→(XI) and (I)→(a)→(d)→(e)→(XI) in Fig. 15A were proposed recently[44,45]. Schoemaker *et al.*[48] also dicussed single-electron oxidation of lignin substructure models by the enzyme.

As for the Cα-Cβ cleavage to produce 4-ethoxy-3-methoxybenzyl alcohol (XXI), two mechanisms were proposed in this chapter: (i) Pathway B in Fig. 4 or pathway B in Fig. 12, and (ii) the pathway of the formation of 2-guaiacoxyethanol (XXVI) (Fig. 14). The latter, mechanism for the formation of (XXVI), involves initial single-electron oxidation of the B-nucleus by the enzyme. On the other hand, Cα-Cβ cleavage may occur on the consequence of initial single-electron oxidation

of the A-nucleus by the enzyme (Fig. 15B), as proposed recently[44,45]. The mechanism is similar to pathway B in Fig. 4 and pathway B in Fig. 12. Reduction of (XXIII) to (XXI) by *P. chrysosporium* was described in Section 1. 1.

As described in Section 1. 1, ^{18}O was incorporated from $^{18}O_2$ into Cα-Cβ cleavage product, 4-ethoxy-3-methoxybenzyl alcohol (XXI) in the degradation of β-O-4 lignin model (V-D') by intact cells of *P. chrysosporium*. The mechanism for the ^{18}O incorporation is not fully elucidated, but it probably occurred *via* the cation radical of the B-nucleus. Lignin peroxidase was suggested to oxidize preferentially the A-nucleus, but not B-nucleus, of 1-(4-ethoxy-3-methoxyphenyl)-2-phenoxy-1, 3-propanediol (III) (Section 2. 2). In the degradation of (III) under $^{18}O_2$ by the intact cells of *P. chrysosporium*, ^{18}O was not incorporated into 4-ethoxy-3-methoxybenzyl alcohol (XXI) (data not shown). According to Tien and Kirk[38], ^{18}O was not incorporated from $^{18}O_2$ into a Cα-Cβ cleavage product, 3, 4-dimethoxybenzaldehyde, in the degradation of 1-(3, 4-dimethoxyphenyl)-2-(2-methoxyphenoxy)-3-phenyl-1-propanol by lignin peroxidase. For the Cα-Cβ cleavage they proposed a mechanism similar to Fig. 15B, although the cation radical was not mentioned.

In conclusion, the O-C_4 cleavage of β-O-4 bond to give the arylglycerol and the formation of 2-guaiacoxyethanol (XXVI) were explained on the consequence of initial single-electron oxidation of the B-nuclei of β-O-4 lignin substructure models by lignin peroxidase (Figs. 14 and 15A). Cα-Cβ cleavage to give 4-ethoxy-3-methoxybenzyl alcohol (XXI) probably occur *via* initial single-electron oxidation of the A- or B-nuclei of the β-O-4 lignin models (Figs. 14 and 15B).

2. Aromatic Ring Cleavage by *Phanerochaete chrysosporium*

Earlier studies of fungus-degraded lignin isolated from decayed wood suggested cleavage of aromatic rings as well as side chains of decayed lignin[4,28,29,35,49–51]. Many degradative reactions of lignin substructure models by intact cells of white-rot fungi, especially *Phanerochaete chrysosporium* and *Coriolus versicolor* were found[3,5–7,11–21,25,26,30,31,34,39,52–63], and most of these reactions were found to be catalyzed by lignin peroxidase (ligninase) of *P. chrysosporium*[36–38,42–45,64–67]. However, products of aromatic ring cleavage were not successfully isolated, and the mechanism of aromatic ring cleavage of lignin by the fungi and its lignin degrading enzymes remained uncertain. In this chapter, first identification of products of aromatic ring cleavage of β-O-4 lignin substructure models by intact cultures of *Phanerochaete chrysosporium* is described.

2. 1 Aromatic Ring Cleavage Products – Part 1 Identification of a Cyclic Carbonate of Arylglycerol

2. 1. 1 Introduction

During the investigation of mechanism for the arylglycerol formation, degradation products of β-O-4 lignin models by *Phanerochaete chrysosporium* were usually acetylated and analyzed by GC-MS as described in Chapter 1. An unknown peak was always detected, which was eluted from the GC column after acetate of the arylglycerol (XI-Ac).

The compound was suspected to be an aromatic ring cleavage product, and it was finally found to be the case. This is the first report on isolation and definite identification of a product of aromatic ring cleavage of β-O-4 lignin substructure models by white-rot fungi[68].

2. 1. 2 Results

After *Phanerochaete chrysosporium* was incubated with arylglycerol-β-guaiacyl ether (I) for 93 hr as described in the previous sections, acetate of cyclic carbonate [(XIV-Ac), about 40 μg, 0.26% (mol product formed/mol initial substrate) × 100] was isolated from the acetate of the culture extract by silica gel TLC separation and iden-

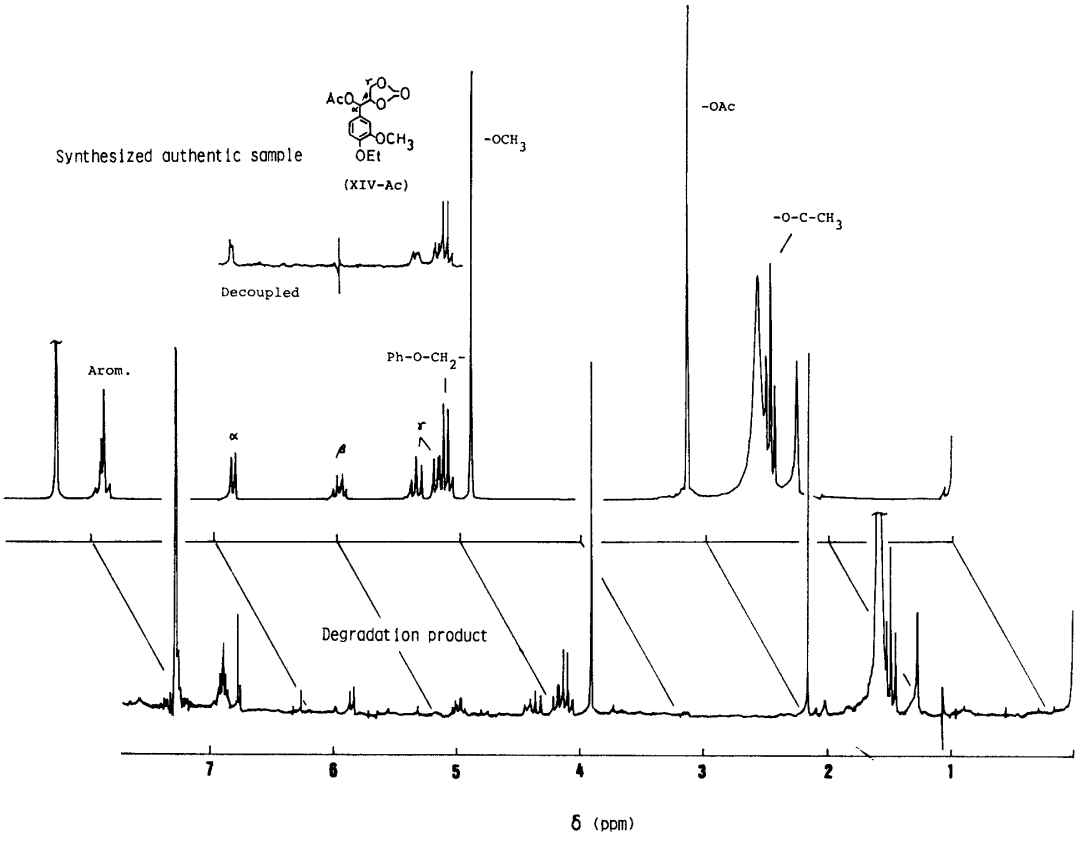

Fig. 16. ^{1}H-NMR spectra of 1-(4-ethoxy-3-methoxyphenyl)-1, 2, 3-propanetriol-2, 3-cyclic carbonate (acetate) (XIV-Ac). Solvent: $CDCl_3$.

tified by comparison of the ^{1}H-NMR and MS spectra with those of synthesized authentic compound (Fig. 16). To prove that the carbonate carbon atom was derived from the aromatic ring carbon of the guaiacyl group of arylglycerol-β-guaiacyl ether (I), arylglycerol-β-[U-*ring*-^{13}C] guaiacyl ether (I-^{13}C) was synthesized and degraded under the same condition. GC-MS analysis of the acetate of the metabolites showed that the mass spectrum of the acetate of cyclic carbonate from (I-^{13}C) exhibited higher molecular ion peak by one mass unit than that of unlabeled authentic compound as shown in Fig. 17. Uninoculated control culture did not catalyze the formation of (XIV) and (XI).

The extract of the culture incubated with β, γ-cyclic carbonate of arylglycerol (XIV) for 32 hr was acetylated and analyzed by GC-MS, and the triacetate of arylglycerol (XI-Ac) was identified by comparison of the mass spectrum [m/z (%): 368(7.8), 308(8.3), 266(9.1), 250(6.4), 248(4.3), 223(6.1), 206(33.4), 181(100)] with that of authentic compound (Fig. 9). However, acetate of arylglycerol (XI-Ac) was also detected in the acetylated extract of uninoculated control culture incubated with β, γ-cyclic carbonate (XIV) for 32 hr.

The acetyl groups of acetate of cyclic carbonate (XIV-Ac) and acetate of arylglycerol (XI-Ac) were derived from the artificial acetylation, because GC-MS analysis showed that the degradation product of arylglycerol-β-guaiacyl ether (I) before the acetylation did not contain the acetates (XIV-Ac) and (XI-Ac).

2. 1. 3 Discussion

The present results proved for the first time that the guaiacyl aromatic ring etherified at the β-position of arylglycerol-β-guaiacyl ether (I) was cleaved to give

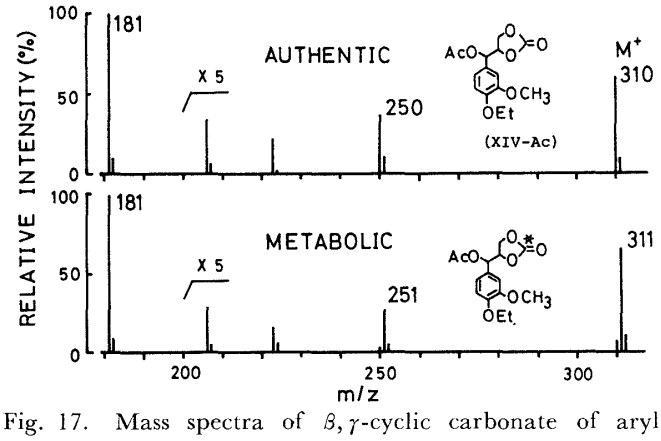

Fig. 17. Mass spectra of β, γ-cyclic carbonate of arylglycerol (acetate) (XIV-Ac). Upper, unlabeled authentic compound; lower, degradation product from arylglycerol-β-[U-*ring*-^{13}C] guaiacyl ether (I-^{13}C). *: ^{13}C.

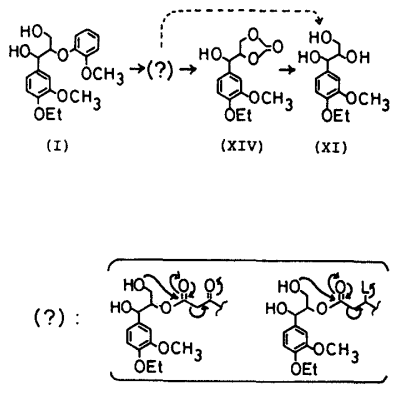

Fig. 18. Formation of β, γ-cyclic carbonate of arylglycerol (XIV) from arylglycerol-β-guaiacyl ether (I) by *Phanerochaete chrysosporium*. (?): Assumed precursors for (XIV). L: Leaving group.

β,γ-cyclic carbonate (XIV) as an aromatic ring cleavage product (Fig. 18).

At the time of first identification of β,γ-cyclic carbonate (XIV)[68], there was no evidence which explains the formation mechanism of β,γ-cyclic carbonate (XIV). *cis,cis*-Muconate, muconolactones and 3-oxoadipate are well known metabolic intermediates of phenols by bacteria and fungi[69]. So, one possibility was that the precursor of β,γ-cyclic carbonate (XIV) was a 3-oxoester or an ester which has a leaving group at 3-position to the ester carboxyl group (*e.g.* a 3-oxoadipate or an ester of muconolactone) (Fig. 18). Subsequently β,γ-cyclic carbonate (XIV) was found to be formed from arylglycerol-β-guaiacyl ether (I) by lignin peroxidase of *P. chrysosporium* (Section 3. 1), and a mechanism for its formation was proposed based on tracer experiments, which will be described in Section 3. 3.

β,γ-Cyclic carbonate (XIV) was partly degraded to arylglycerol (XI) in the intact culture of *P. chrysosporium* and the corresponding uninoculated culture, and is, threfore, one of the precursor of arylglycerol (XI).

2. 2 Aromatic Ring Cleavage Products – Part 2 Other Aromatic Ring Cleavage Products

2. 2. 1 Introduction

Besides β,γ-cyclic carbonate (XIV) (Section 2. 1)[68], several esters of arylglycerol were identified as products of aromatic ring cleavage of β-O-4 lignin substructure model dimers by *Phanerochaete chrysosporium*[70]. Substituent effects were observed in their formation: Substituents on the B-rings of the β-O-4 type models influenced the mode of formation of the ring cleavage products.

2. 2. 2 Results

1-(4-Ethoxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)-1, 3-propanediol (I) (2 mg), 2-(2, 6-dimethoxyphenoxy)-1-(4-ethoxy-3-methoxyphenyl)-1, 3-propanediol (II) (2 mg), 2-(2, 6-dimethoxy [U-*ring*-^{13}C] phenoxy)-1-(4-ethoxy-3-methoxyphenyl)-1, 3-propanediol (II-^{13}C) (*ca.* 1 mg), 1-(4-ethoxy-3-methoxyphenyl)-2-phenoxy-1, 3-propanediol (III) (2 mg) and 1-(4-ethoxy-3-methoxyphenyl)-2-(4-formyl-2-methoxyphenoxy)-1, 3-propanediol (IV) (2 mg) were incubated with *P. chrysosporium* (87–94 hr) as described in the previous sections. Degradation products were extracted and acetylated. The acetylated products were dissolved in 100 μl of acetone, and 1 μl of the acetone solution was injected into GC-MS. In a separate run, 17.2 mg of arylglycerol-β-guaiacyl ether (I) was incubated with the fungus for 93 hr. The degradation products were extracted and acetylated as above. The products of acetylation was submitted to silica gel TLC, and acetate of 1-(4-ethoxy-3-methoxyphenyl)-1, 2, 3-propanetriol-1, 2-cyclic carbonate (XV-Ac) was isolated.

Fig. 19 shows the total ion chromatograms of the acetylated products. The following products were identified by comparison of the mass spectra with those of

Table 3. Relative intensity of the important fragment ions of the metabolic products (acetate)

Product	Mass spectral data, m/z (Relative intensity, %)
Metabolic products derived from (I)	
(XI-Ac)	368 (M^+, 6.4), 308 (7.9), 233 (4.7), 206 (32.7), 181 (100), 153 (9.9), 151 (11.1)
(XIII-Ac)	324 (M^+, 6.2), 179 (100), 151 (57.6), 123 (12.4)
(XIV-Ac)	310 (M^+, 10.3), 223 (4.0), 206 (3.5), 181 (100), 153 (14.8), 151 (9.7)
(XV-Ac)	310 (M^+, 84.2), 206 (46.5), 179 (22.7), 178 (40.5), 177 (20.0) 165 (13.0), 151 (100)
(XVI-Ac)	354 (M^+, 9.0), 294 (4.7), 223 (5.9), 206 (23.1), 181 (100), 153 (12.4), 151 (11.6)
(XXI-Ac)	224 (M^+, 62.4), 196 (5.2), 182 (16.7), 165 (25.1), 154 (29.6), 153 (11.8), 151 (12.1), 139 (8.4), 138 (9.7), 137 (100)
Metabolic products derived from (II)	
(XI-Ac)	368 (M^+, 8.2), 308 (5.0), 223 (5.5), 206 (19.4), 181 (100), 153 (9.5), 151 (8.0)
(XIII-Ac)	324 (M^+, 5.9), 179 (100), 151 (51.7), 123 (10.2)
(XIV-Ac)	310 (M^+, 10.4), 223 (4.1), 206 (2.6), 181 (100), 153 (15.2), 151 (12.7)
(XV-Ac)	310 (M^+, 78.6), 206 (54.4), 179 (17.3), 178 (41.2), 177 (20.7), 165 (8.5), 151 (100)
(XVI-Ac)	354 (M^+, 8.2), 294 (4.0), 223 (4.6), 206 (18.6), 181 (100), 153 (11.9), 151 (10.2)
(XXI-Ac)	224 (M^+, 50.7), 196 (5.4), 182 (15.2), 165 (23.5), 154 (27.8), 153 (12.1), 151 (13.5), 139 (9.9), 138 (10.5), 137 (100)
(XVII-Ac)	412 (M^+, 8.6), 352 (2.6), 223 (3.9), 207 (14.7), 206 (17.9), 181 (100)
Metabolic products derived from (III)	
(XI-Ac)	368 (M^+, 7.5), 308 (3.0), 223 (4.1), 206 (16.5), 181 (100), 153 (7.3), 151 (7.6)
(XIII-Ac)	324 (M^+, 5.8), 179 (100), 151 (52.6), 123 (9.8)
(XXI-Ac)	224 (M^+, 54.9), 196 (4.8), 182 (16.4), 165 (24.3), 154 (28.3), 153 (12.4), 151 (13.9), 139 (8.2), 138 (9.4), 137 (100)
Metabolic products derived from (IV)	
(XI-Ac)	368 (M^+, 6.0), 308 (7.1), 223 (5.8), 206 (29.8), 181 (100), 153 (11.1) 151 (20.0)
(XIII-Ac)	324 (M^+, 5.3), 179 (100), 151 (58.3), 123 (11.6)
(XIV-Ac)	310 (M^+, 12.0), 223 (3.8), 206 (4.3), 181 (100), 153 (15.8), 151 (12.0)
(XV-Ac)	310 (M^+, 60.8), 206 (39.5), 179 (23.3), 178 (32.9), 177 (17.5), 165 (14.0), 151 (100)
(XXI-Ac)	224 (M^+, 60.9), 196 (6.2), 182 (19.3), 165 (25.6), 154 (31.9), 153 (13.6), 151 (16.1), 139 (9.4), 138 (10.3), 137 (100)
Authentic compounds	
Compound	Mass spectral data, m/z (Relative intensity, %)
(XI-Ac)	368 (M^+, 5.9), 308 (7.6), 223 (4.1), 206 (29.8), 181 (100), 153 (10.0), 151 (10.6)
(XIII-Ac)	324 (M^+, 5.9), 179 (100), 151 (54.8), 123 (10.5)
(XIV-Ac)	310 (M^+, 11.1), 223 (3.8), 206 (2.0), 181 (100), 153 (14.5), 151 (8.5)

Table 3. (cont'd)

(XV-Ac)	310 (M^+, 72.5), 206 (52.4), 179 (16.9), 178 (41.2), 177 (19.2), 165 (7.9), 151 (100)
(XVI-Ac)	354 (M^+, 7.6), 294 (3.7), 223 (4.1), 206 (18.6), 181 (100), 153 (9.7), 151 (6.0)
(XXI-Ac)	224 (M^+, 55.0), 196 (6.0), 182 (18.2), 165 (24.7), 154 (31.7), 153 (13.1), 151 (10.8), 139 (8.4), 138 (9.8), 137 (100)
(XVII-Ac)	412 (M^+, 12.1), 352 (3.4), 223 (5.0), 207 (14.6), 206 (18.8), 181 (100)

authentic compounds (Table 3).

Arylglycerol (XI), dihydroxypropiophenone (XIII), cyclic carbonates (XIV) and (XV), γ-formate (XVI) and Cα-Cβ cleavage product (XXI) were formed from arylglycerol-β-(2, 6-dimethoxyphenyl) ether (II) and identified (as acetates) (Table 3, Fig. 19). (II) was completely degraded during the incubation. In the degradation of arylglycerol-β-phenyl ether (III), (XXI) was detected as a product (Table 3). As for arylglycerol-β-guaiacyl ether (I) and arylglycerol-β-vanillin ether (IV), the formation of compounds (XI), (XIII), (XIV), (XV) and (XXI) was observed (Table 3, Fig. 19). Furthermore, the identification of α, β-cyclic carbonate (XV) from arylglycerol-β-guaiacyl ether (I) was confirmed by ^{1}H-NMR analysis. Fig.

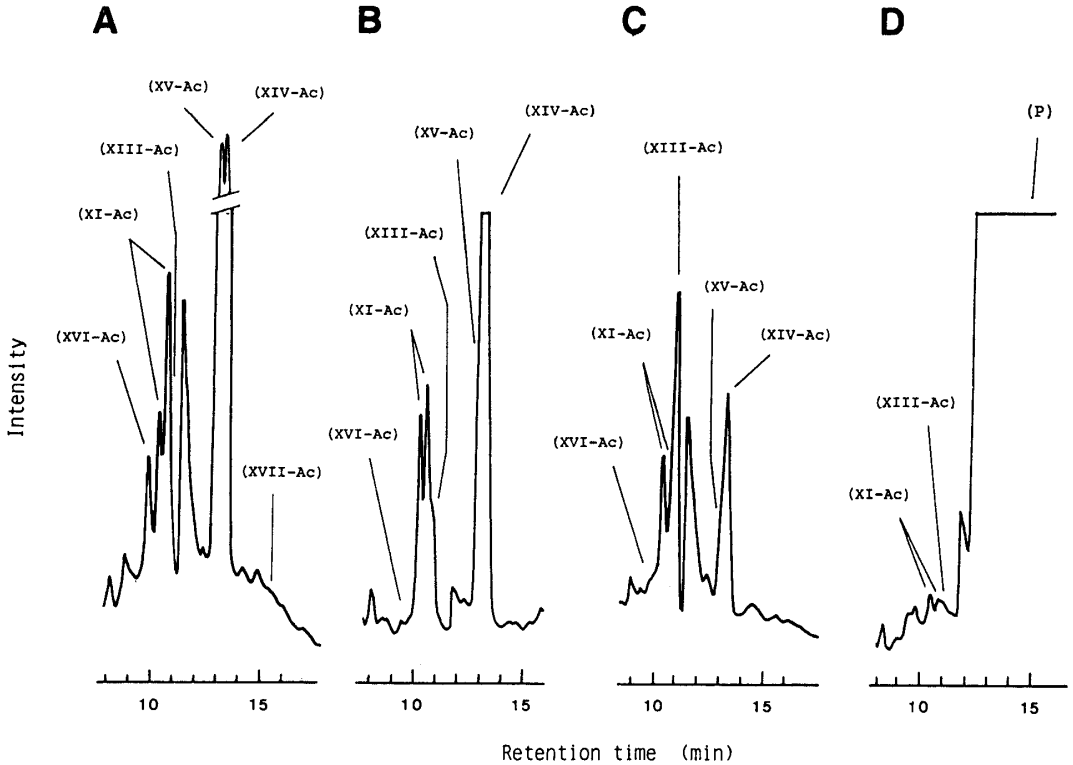

Fig. 19. Total ion chromatograms of the acetylated products of the degradation of β-O-4 lignin models, (I), (II), (III) and (IV), by intact cells of *Phanerochaete chrysosporium*. A, B, C and D: Products of the degradation of (II), (I), (IV) and (III), respectively. (P) in D: 1-(4-ethoxy-3-methoxyphenyl)-3-hydroxy-2-phenoxypropane-1-one (date not shown).

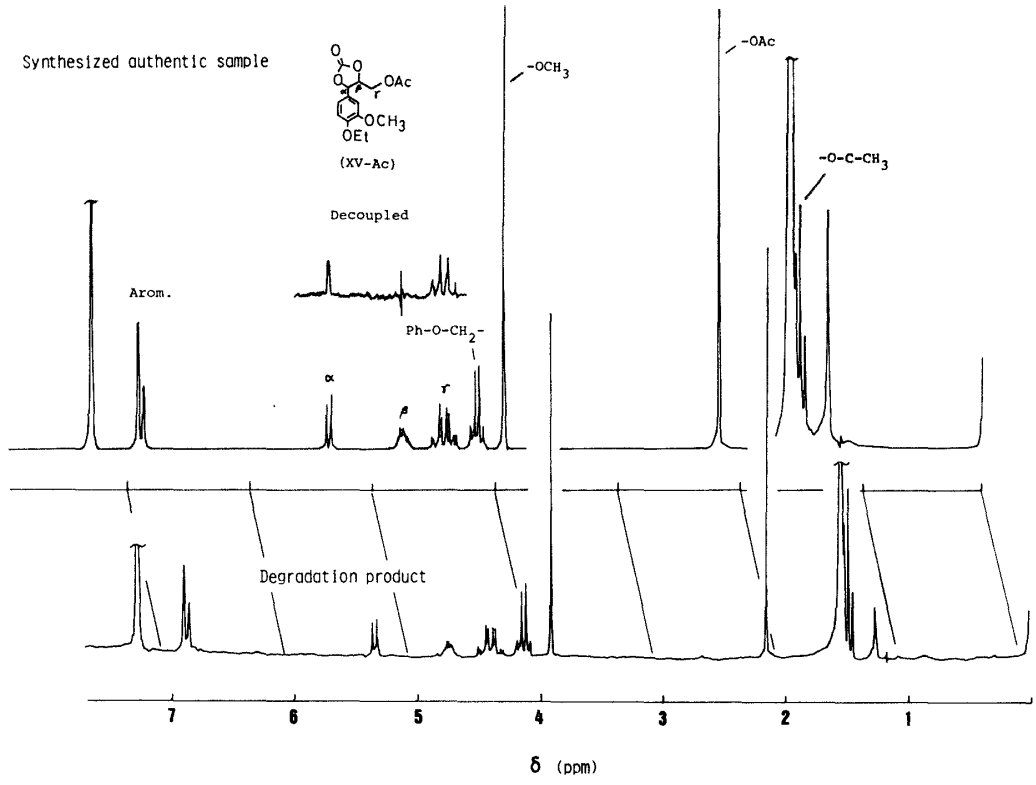

Fig. 20. ^{1}H-NMR spectra of 1-(4-ethoxy-3-methoxyphenyl)-1, 2, 3-propanetriol-1, 2-cyclic carbonate (acetate) (XV-Ac). Solvent: $CDCl_3$.

20 shows ^{1}H-NMR spectra of acetate of the degradation product (XV-Ac) and the authentic sample. Although the corresponding peaks were not observed in the total ion chromatograms as shown in Fig. 19, background subtraction of mass spectra indicated the formation of trace amounts of the following products (Table 3): γ-Formate (XVI) from arylglycerol-β-guaiacyl ether (I), methyl oxalate (XVII) from arylglycerol-β-(2, 6-dimethoxyphenyl) ether (II), arylglycerol (XI) and dihydroxypropiophenone (XIII) from arylglycerol-β-phenyl ether (III). Moreover mass chromatographic analysis of the acetylated products of degradation of arylglycerol-β-vanillin ether (IV) suggested the formation of γ-formate (XVI). The substrates (I), (III) and (IV) were not completely degraded during the incubation. Uninoculated control culture did not give any of the products.

The carbonyl carbon atoms of (XIV), (XV), (XVI), and (XVII) were found to be derived from the 2, 6-dimethoxyphenyl nucleus of arylglycerol-β-(2, 6-dimethoxyphenyl) ether (II) based on tracer experiment with ^{13}C. Fig. 21 shows the mass spectra of (XIV-Ac), (XV-Ac) and (XVI-Ac) from (II-^{13}C). The spectra showed that the molecular ion peaks of the products were higher by one mass unit than those of unlabeled authentic compounds. Fig. 22 shows the mass chromatograms of the molecular ion region and important fragment ions of (XVII-Ac) produced from

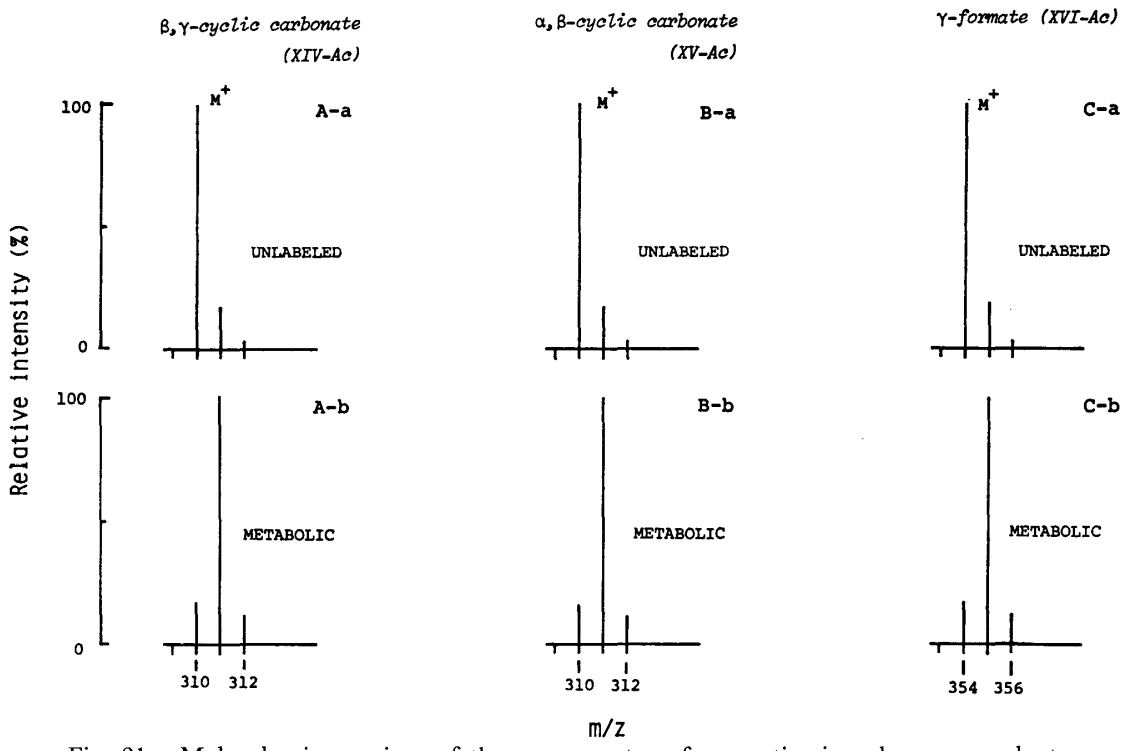

Fig. 21. Molecular ion regions of the mass spectra of aromatic ring cleavage products (acetates) in the degradation of (II-^{13}C) by *Phanerochaete chrysosporium* and their authentic samples. A-a, B-a and C-a are molecular ion regions of the mass spectra of unlabeled authentic samples. A-b, B-b and C-b are those of products of degradation of (II-^{13}C), B-ring of which is uniformly labeled with ^{13}C (^{13}C: 90 atom%), by intact cells of *Phanerochaete chrysosporium*.

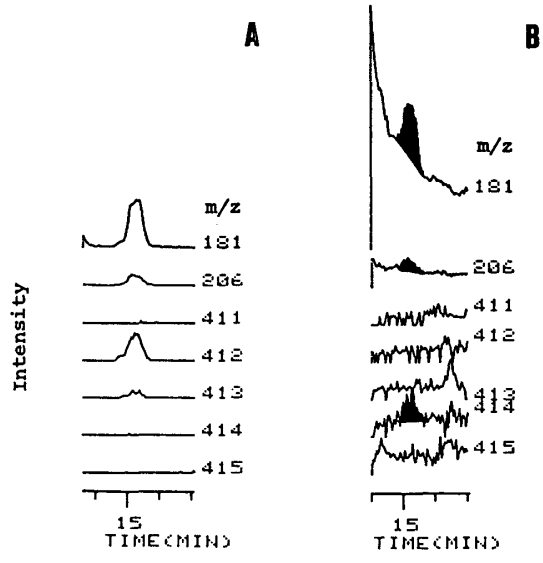

Fig. 22. Mass chromatograms of molecular ion regions and important fragment ions of acetate of methyl oxalate (XVII-Ac). A: Unlabeled authentic sample (molecular weight: 412). B: Product of degradation of (II-^{13}C) by *Phanerochaete chrysosporium*.

(II-^{13}C). The chromatogram showed that the molecular ion peak of the product (m/z, 414) was higher by two mass units than that of unlabeled authentic sample (m/z, 412).

Preliminary quantification by means of stable isotope dilution procedure showed that the yields of 4-ethoxy-3-methoxybenzyl alcohol (XXI) and arylglycerol (XI) (sum of *erythro* and *threo* isomers) were 167 μg and 18 μg, respectively, after 90 hr-incubation of (I) (2.0 mg) with the culture (20 ml) of *P. chrysosporium*, where the incubation conditions were the same as in the present study.

2. 2. 3 Discussion

Study of the degradation of arylglycerol-β-guaiacyl ether (I) by intact cells of *P. chrysosporium* showed the formation of aromatic ring cleavage product, β, γ-cyclic carbonate (XIV) as described in the precedent section[68]. β, γ-Cyclic carbonate (XIV) was also formed from arylglycerol-β-guaiacyl ether (I), arylglycerol-β-(2, 6-dimethoxyphenyl) ether (II) and arylglycerol-β-syringaldehyde ether (VI) in the culture of *C. versicolor*, and γ-formate (XIV) was identified as another aromatic ring cleavage product in the degradation of arylglycerol-β-(2, 6-dimethoxyphenyl) ether (II) and arylglycerol-β-syringaldehyde ether (VI), which have two methoxyl substituents one the B-ring, by *C. versicolor*[71]. In addition, recent studies of the degradation of β-O-4 models by lignin peroxidase of *P. chrysosporium* showed that the enzyme catalyzed aromatic ring cleavage of (I) and (II) (Section 3. 1)[46,47], to yield β, γ-cyclic carbonate (XIV), γ-formate (XVI) and novel aromatic ring cleavage products, α, β-cyclic carbonate (XV) and methyl oxalate (XVII) which were not detected in the previous investigations with ligninolytic cultures of both the fungi. The identification of products of aromatic ring cleavage by lignin peroxidase will be described in Chapter 3. The present investigation confirmed that all the products of aromatic ring cleavage by lignin peroxidase, α, β-cyclic carbonate (XV), γ-formate (XVI) and methyl oxalate (XVII) as well as β, γ-cyclic carbonate (XIV) were also formed in the degradation of the β-O-4 lignin model dimers by intact cells of *P. chrysosporium*. These results strongly suggest that aromatic ring cleavage (formation of (XIV), (XV), (XVI) and (XVII) from the β-O-4 lignin substructure model dimers) in the culture of *P. chrysosporium* was catalyzed by lignin peroxidase.

In the present study, the degradation of β-O-4 lignin models with different aromatic substituents on the B-nucleus by intact cells of *P. chrysosporium* was examined. The total ion chromatograms (Fig. 19) showed that the number of methoxyl groups considerably influenced the reactivity of the β-O-4 models. The same results were obtained recently based on detailed quantification of the products[72]. In the degradation of arylglycerol-β-(2, 6-dimethoxyphenyl) ether (II) which has two methoxyl substituents on the B-nucleus by the intact cells, arylglycerol (XI), dihydroxypro-

piophenone (XIII), β,γ-cyclic carbonate (XIV), α,β-cyclic carbonate (XV), formate (XVI), α,β-cleavage product (XXI) and trace amounts of methyl oxalate (XVII) were detected. For arylglycerol-β-guaiacyl ether (I), which has one methoxyl substituent on the B-nucleus, formation of (XI), (XIII), (XIV), (XV), (XXI) and trace amounts of (XVI) were detected. As for the degradation of arylglycerol-β-vanillin ether (IV) which has one methoxyl and one formyl substituents on the B-nucleus, (XI), (XIII), (XIV), (XV) and (XXI) were formed as products, and formation of trace amounts of γ-formate (XVI) was also suggested by mass chromatography. Thus, yields of γ-formate (XVI) from (I) and (IV) were small, whereas that from (II) was significant. In the degradation of arylglycerol-β-phenyl ether (III), which has no methoxyl substituent on the B-nucleus, by the fungus, (XXI) and trace amounts of (XI) and (XIII) were produced. However, formation of aromatic ring cleavage products could not be detected. The relationship between the products and the substrates is in accord with the results with lignin peroxidase of *P. chrysosporium*[46,47], which will be described in Chapter 3, and intact cells of *C. versicolor*[71]. Miki *et al.*[44] reported that arylglycerol (XI) was not detected in the degradation of another β-O-4 model, 1-(4-ethoxy-3-methoxyphenyl)-2-(4-hydroxymethylphenoxy)-1, 3-propanediol analogous to (III), by lignin peroxidase of *P. chrysosporium*. Since evidence has been provided that lignin peroxidase catalyzes single-electron oxidation of the aromatic nuclei to the unstable cation radicals[40,45], it seems to be reasonable that the A-nucleus of arylglycerol-β-phenyl ether (III) having two electron donating alkoxyl groups was preferentially oxidized by lignin peroxidase than the B-nucleus having one alkoxyl group resulting in the formation of 4-ethoxy-3-methoxybenzyl alcohol (XXI) *via* 4-ethoxy-3-methoxybenzaldehyde (XXIII) (pathway B, Fig. 15). Reduction of (XXIII) to (XXI) by the fungus was described in Section 1. 1.

Benzaldehydes are formed in Cα-Cβ cleavage of β-O-4 and β-1 lignin substructure models and lignin by lignin peroxidase[36–38,42–46]. Accordingly arylglycerol-β-vanillin ether (IV) represents lignin degradation intermediates more accurately than (I), (II) and (III), which have no substituent *para* to the β-O-4 bond. Recently Kirk *et al.*[45] mentioned that a β-O-4 model compound (X) which is Cα-carbonyl analog of (IV) was not a substrate for lignin peroxidase, suggesting that the electron withdrawing formyl and carbonyl groups render both the aromatic nuclei (A and B nuclei) resistant to oxidation by lignin peroxidase. Present study using intact cells of *P. chrysosporium*, however, showed that the formyl substituent on the B-nucleus of arylglycerol-β-vanillin ether (IV) did not inhibit fungal attack on the B-ring, the formation of arylglycerol (XI), dihydroxypropiophenone (XIII), cyclic carbonates (XIV) and (XV) at least qualitatively by intact cells of the fungus, although evi-

dences were provided that formation of the products from (I) and (II) were catalyzed by lignin peroxidase (Section 3. 1).

3. Aromatic Ring Cleavage by Lignin Peroxidase of *Phanerochaete chrysosporium*

The white-rot fungi *Phanerochaete chrysosporium* and *Coriolus versicolor* cause various oxidative degradations of lignin and lignin substructure model compounds[3,4,39,68,71,73,74]. Some of the reactions such as Cα-Cβ and β-O-4 bond cleavage were found to be catalyzed by an extracellular lignin-degrading enzyme (lignin peroxidase, ligninase) of *P. chrysosporium*[36–38,42–45,64–67]. However, it is not certain yet whether aromatic ring cleavage of lignin and lignin substructure models are also catalyzed by the same lignin peroxidase, although Leisola *et al.*[75] recently reported aromatic ring cleavage of veratryl alcohol by the extracellular enzyme system of *P. chrysosporium.*

The present investigation reports for the first time the evidence that β-O-4 lignin substructure models are degraded by lignin peroxidase of *P. chrysosporium,* yielding aromatic ring cleavage products, and the mechanisms for the ring cleavage are proposed.

3. 1 Aromatic Ring Cleavage by Lignin Peroxidase

3. 1. 1 Introduction

Intact cells of *Phanerochaete chrysosporium* catalyzed aromatic ring cleavage of β-O-4 lignin substructure model compounds as described in Chapter 2. Conventional enzymes responsible for aromatic ring cleavage are dioxygenases, such as protocatechuic acid 3, 4-dioxygenase or catechol dioxygenase[69]. However, the enzyme catalyzing the aromatic ring cleavage of lignin substructure models was found to be lignin peroxidase[46,47].

3. 1. 2 Results

Enzyme Production and Purification

Lignin peroxidase (ligninase) was a generous gift from Nagase Biochemicals, Ltd. (Fukuchiyama, Kyoto, Japan), which was prepared by the modified method of Tien and Kirk[38,76] from the culture filtrate of *Phanerochaete chrysosporium* Burds. (ME-446). The enzyme protein was concentrated by ultrafiltration from the culture filtrate (88 hr cultures). Enzyme activity (International Unit, I.U.) was assayed by spectrometric quantification of veratraldehyde (ε_{310}=9,300 $M^{-1}cm^{-1}$) formed on oxidation of veratryl alcohol at 36°C[38].

Product Identification

Reaction mixture (3.3 ml) contained 60 μl of 25 mM H_2O_2, 1 μmol of model substrate in 30 μl of CH_3OH, 15 μl of lignin peroxidase (0.4–1 I.U.), and 3195 μl of 100 mM sodium tartrate buffer (pH 3.0). The enzymatic reaction was started

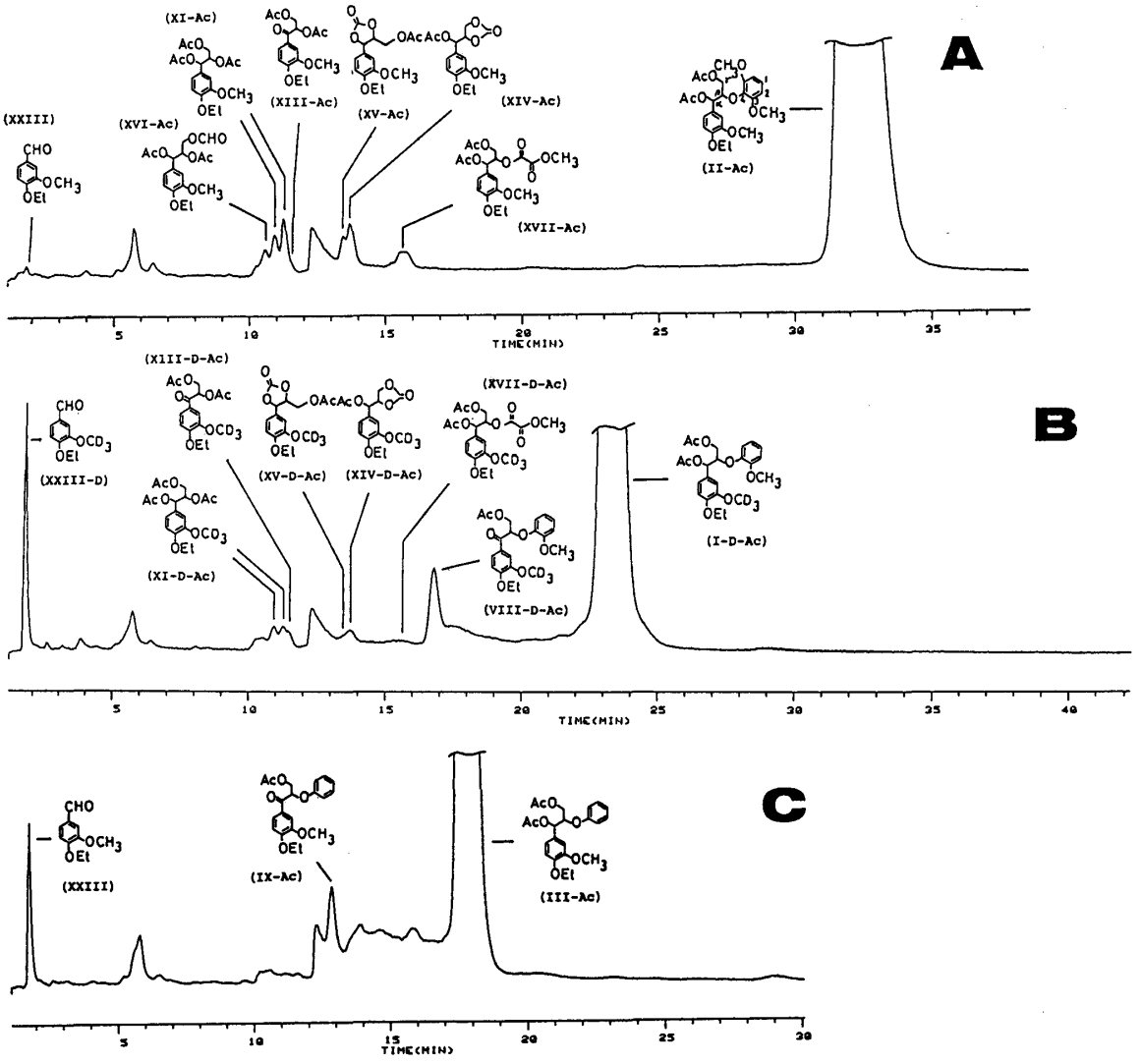

Fig. 23. Total ion chromatograms of reaction products (acetate). (A), (B) and (C): Degradation products (acetate) from (II), (I-D) and (III), respectively.

by the addition of lignin peroxidase into the mixture, which was incubated for 30 min at 37°C under air. The reactions were terminated by extraction with ethyl acetate (15 ml×2). Products were acetylated (Ac_2O/pyridine=1:1, by volume in EtOAc, 15 hr, room temp.) and analyzed by gas chromatography-mass spectrometry (GC-MS). GC-MS analysis (Fig. 23) of acetate of degradation products of aryl-glycerol-β-(2, 6-dimethoxyphenyl) ether (II) showed formation of aromatic ring cleavage products, β,γ-cyclic carbonate (XIV-Ac) [MS m/z (%): 310(M^+, 14.2), 223 (3.5), 206(4.4), 181(100), 153(11.6), 151(10.6)], α,β-cyclic carbonate (XV-Ac) [MS m/z (%): 310(M^+, 79.7), 206(49.9), 179(19.6), 178(39.9), 177(29.3), 165(19.9), 151(100)], and γ-formate (XVI-Ac) [MS m/z (%): 354(M^+, 8.7), 294(4.5), 223(4.6), 206(18.8), 181(100), 153(8.8), 151(8.5)] as well as Cα-Cβ cleavage product (XXIII)

[MS m/z (%): 180(M^+, 48.9), 152(44.7), 151(100)], arylglycerol (XI-Ac) [MS m/z (%): 368(M^+, 8.0), 308(4.3), 266(1.6), 250(1.8), 248(1.0), 223(5.1), 206(18.8), 181 (100)], and dihydroxypropiophenone (XIII-Ac) [MS m/z (%): 324(M^+, 8.7), 264 (6.4), 222(5.2), 191(12.9), 179(100), 151(54.3), 123(14.1)]. The mass spectra and retention times in GC-MS of the above products were identical to those of the synthesized authentic samples. Furthermore a product which has the mass spectrum [MS m/z (%): 412(M^+, 12.1), 352(3.4), 223(5.0), 207(14.6), 206(18.8), 181(100)] was detected and finally identified as methyl oxalate of 1-(4-ethoxy-3-methoxyphenyl)-1, 2, 3-propanetriol (XVII-Ac), because compound (XVII-Et), diethyl ether analog of (XVII), [MS m/z (%): 384(M^+, 5.3), 209(100), 181(14.4), 153(8.3)] was also produced from diethyl ether of (II), (II-Et) (Fig. 24), and identified by comparison of the mass spectrum and the retention time (capillary GC-MS) with those of the synthesized authentic compound.

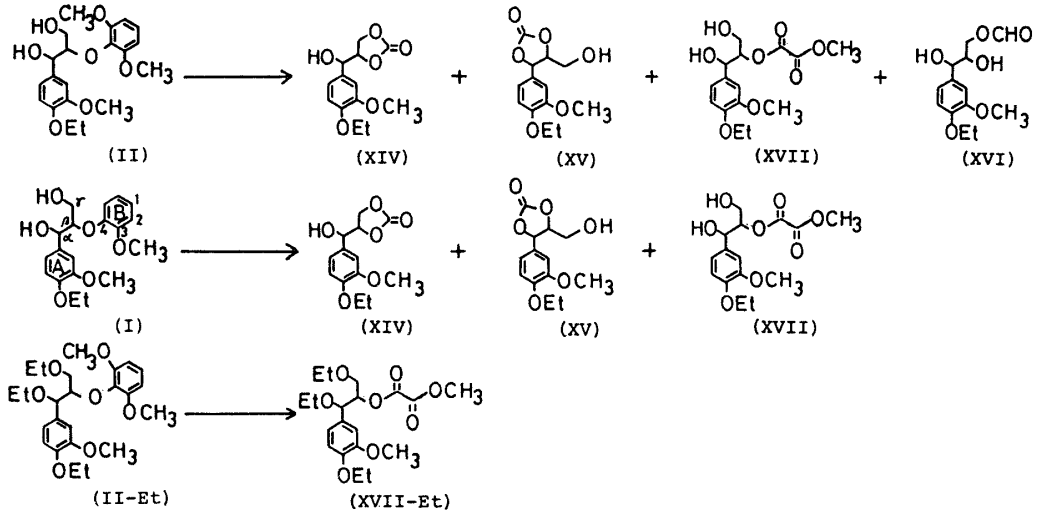

Fig. 24. Products of aromatic ring cleavage of β-O-4 dimers by lignin peroxidase. As for (XVII), position of oxalyl group was assigned tentatively at the β-position based on the identification of (XVII-Et) from (II-Et). Et=CH_2CH_3.

When [OCD_3] arylglycerol-β-guaiacyl ether (I-D) was degraded by the enzyme system, [OCD_3] arylglycerol (XI-D'), [OCD_3]-β, γ-cyclic carbonate (XIV-D), [OCD_3]-α, β-cyclic carbonate (XV-D), methyl oxalate of [OCD_3] arylglycerol (XVII-D), 4-ethoxy-3-([$^2H_3$] methoxy) benzaldehyde (XXIII-D), [OCD_3] dihydroxypropiophenone (XIII-D), and [OCD_3]-α-oxoarylglycerol-β-guaiacyl ether (VIII-D) were also identified as products by comparison of their mass spectra (as acetates except for (XXIII-D)) and retention times with those of authentic samples (Fig. 23). (VIII-D-Ac): MS m/z (%): 331(M^+-$C_2H_4O_2$, 20.2), 182(100), 180(25.1), 154(66.0).

Arylglycerol-β-phenyl ether (III) having no methoxyl group on β-aryl group

(B-ring, see also Fig. 23) was also degraded by lignin peroxidase/H_2O_2 system. The acetylated enzymatic reaction products were analyzed by GC-MS. Only 4-ethoxy-3-methoxybenzaldehyde (XXIII) and α-oxoarylglycerol-β-phenyl ether (IX) were identified as degradation products by comparison of their mass spectra ((IX): as the acetate (IX-Ac)) and retention times with those of authentic samples (Fig. 23). (IX-Ac): MS m/z (%), 298(M^+-$C_2H_4O_2$, 40.4), 179(81.0), 177(42.0), 151(100). Arylglycerol (XI-Ac), β,γ-cyclic carbonate (XIV-Ac), α,β-cyclic carbonate (XV-Ac), γ-formate (XVI-Ac), methyl oxalate of arylglycerol (XVII-Ac), and dihydroxypropiophenon (XIII-Ac) were not detected.

Product Quantification

Table 4 summarizes the results of quantitative analysis based on stable isotope dilution method. The above described cleavage reactions were completely dependent on the presence of H_2O_2.

Table 4 Yield of products from substrates (I-D) and (II-D)

Substrates		Products (nmol)					
		(XXIII-D)	(XI-D)	(XIII-D)	(XIV-D)	(XV-D)	(VIII-D)
(I-D)	Complete*	130	14	8.2	3.3	1.1	15
	$-H_2O_2$**	1.3	ND	ND	ND	ND	1.4
	Denatured***	1.1	ND	ND	ND	ND	ND
(II-D)	Complete*	0.9	26	0.9	13	11	—
	$-H_2O_2$**	0.7	2.0	ND	1.4	0.6	—
	Denatured***	0.2	ND	ND	ND	ND	—

*: Complete system consisted of 1 μmol substrate (I-D) or (II-D), H_2O_2, lignin peroxidase, and the buffer. **: The same system as the complete system except that H_2O_2 was replaced with distilled water. The '$-H_2O_2$ system' also exhibited weak activity of oxidation of veratryl alcohol. ***: The same system as the complete system except that lignin peroxidase was replaced with the enzyme denatured on boiling at 100°C for 5 min. The 'denatured system' did not catalyze the formation of (XVII-D) from (I-D) and (II-D), and 1-[4-ethoxy-3-([$^2H_3$]methoxy) phenyl]-1, 2, 3-propanetriol-3-formate from (II-D). ND: not detected.

^{13}C-tracer experiments

Arylglycerol-β-[U-*ring*-^{13}C] guaiacyl ether (I-^{13}C) and arylglycerol-β-(2, 6-dimethoxy [U-*ring*-^{13}C] phenyl) ether (II-^{13}C) were degraded by the lignin peroxidase/H_2O_2 system. GC-MS analysis (mass chromatography) of acetates of the products (Fig. 25) showed that β,γ-cyclic carbonate (XIV-Ac), α,β-cyclic carbonate (XV-Ac), γ-formate (XVI-Ac) and methyl oxalate (XVII-Ac) were formed as aromatic ring cleavage products by the enzyme system: Molecular ion peaks of the cyclic carbonates and the formate from (I-^{13}C) and/or (II-^{13}C) were m/z 311 and 355, respectively, one mass unit higher than those of the products from unlabeled (II),

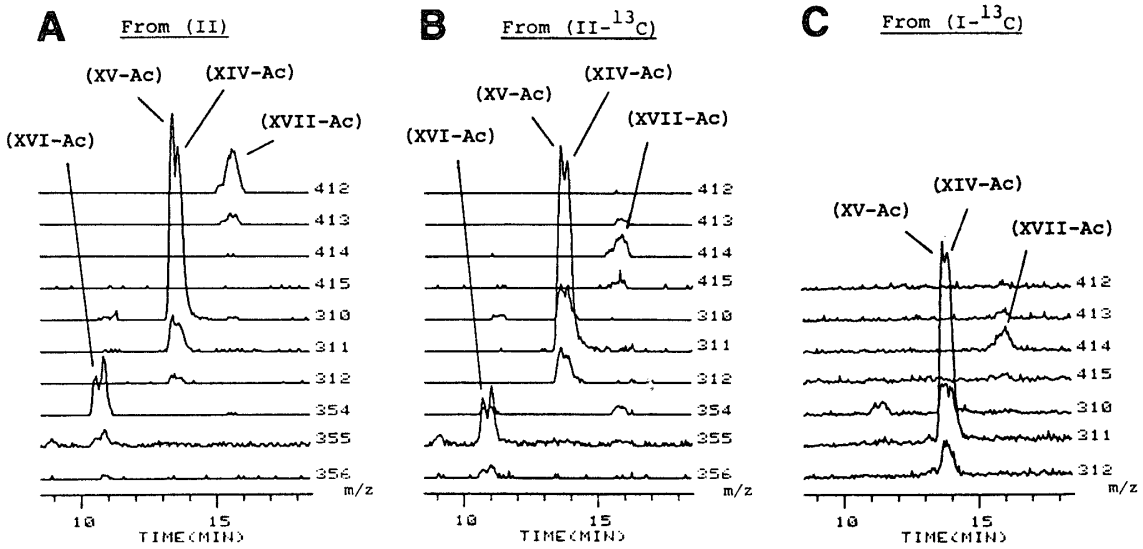

Fig. 25. Mass chromatograms of molecular ion peaks of degradation products from (II), (II-^{13}C) and (I-^{13}C) by lignin peroxidase/H_2O_2 system. (II): Isotopically unmodified compound, (I-^{13}C): 1-(4-ethoxy-3-methoxyphenyl)-2-(2-methoxy-[U-*ring*-^{13}C] phenoxy)-1, 3-propanediol (^{13}C: 90 atom%, statistically uniform labeling), (II-^{13}C): 2-(2, 6-dimethoxy [U-*ring*-^{13}C] phenoxy)-1-(4-ethoxy-3-methoxyphenyl)-1, 3-propanediol (^{13}C: 90 atom%, statistically uniform labeling). A: Products from (II), molecular ion peaks of (XVI-Ac), (XV-Ac), (XIV-Ac) and (XVII-Ac) are m/z 354, 310, 310 and 412, respectively. B, C: Products from (II-^{13}C) and (I-^{13}C), respectively; molecular ion peaks of (XVI-Ac), (XV-Ac), (XIV-Ac) and (XVII-Ac) are 355, 311, 311 and 414, respectively.

and molecular ion peak of the methyl oxalate from both (I-^{13}C) and (II-^{13}C) was m/z 414, 2 mass units higher than that from unlabeled (II) (Fig. 25).

3. 1. 3 Discussion

Previous studies showed that the aromatic rings of β-O-4 lignin substructure model dimers were cleaved by ligninolytic cultures of *Phanerochaete chrysosporium* (Chapter 2)[68,70] and *Coriolus versicolor*[71]. β, γ-Cyclic carbonate (XIV) was identified as a product of aromatic ring cleavage of arylglycerol-β-guaiacyl ether (I) and arylglycerol-β-(2, 6-dimethoxyphenyl) ether (II) by both fungi. γ-Formate (XVI) was also identified as products of (II) by both fungi. Furthermore, some of the ring cleavage products were very recently identified as products of degradation of (I) by intact cells of *Coriolus hirsutus*[77]. Although Leisola *et al.*[75] recently reported aromatic ring cleavage of a monomeric aromatic compound, veratryl alcohol, by crude extracellular ligninase (lignin peroxidase) preparation, this is the first paper on the demonstration that the lignin peroxidase catalyzed in the presence of H_2O_2 aromatic ring cleavage of β-O-4 lignin substructure models: Cylcic carbonates, formate, and methyl oxalate of arylglycerols were formed as products of aromatic ring cleavage of β-O-4 lignin substructure models, (I-D), (II) and (II-Et) (Fig. 24)[46,47]. ^{13}C-tracer

experiments confirmed that the carbonyl carbons of the products are derived from β-aryl group (B-ring). Since lignin peroxidase produces aryl cation radicals from methoxylated aromatic substrates[40,45], the aromatic ring cleavage probably proceeds *via* the aryl cation radicals. Detailed mechanism for the ring cleavage will be discussed in Section 3. 3.

Recently, Kirk *et al.*[45] reported that the major consequence of ligninase (lignin peroxidase)-catalyzed oxidation of 1-(3, 4-dimethoxyphenyl)-2-(2-methoxyphenoxy)-1, 3-propanediol (4-methoxy analog of (I)) was Cα-Cβ cleavage to give veratraldehyde. The present results of quantification indicated that a major product was Cα-Cβ cleavage product (XXIII) in the degradation of arylglycerol-β-guaiacyl ether (I-D) by the lignin peroxidase, whereas arylglycerol-β-(2, 6-dimethoxyphenyl) ether (II) was degraded by preferential attack on higher alkoxylated B-ring yielding no significant amount of Cα-Cβ cleavage product (XXIII), but giving mainly B-ring cleavage products and O-C_4 cleavage product (XI). Evidence has been provided that lignin peroxidase oxidizes methoxylated aromatic ring to give aryl cation radicals[40,45]. Since alkoxyl groups are electron-donating, it seems to be reasonable that higher alkoxylated aromatic rings are preferentially attacked by lignin peroxidase to give aromatic ring cleavage products and arylglycerol identified in the present investigation.

The lignin peroxidase is now known to catalyze Cα-Cβ cleavage of β-O-4 and β-1 lignin substructure models and lignin[36–38,42–46,64–67] and O-C_4 cleavage of β-O-4 bond to give arylglycerol such as (XI)[37,42–47,65]. It is interesting and noteworthy that lignin peroxidase catalyzes both aromatic ring cleavage and cleavage of propyl side chain of lignin substructure models and that aromatic ring cleavage of lignin substructure models does not need conventional dioxygenases.

3. 2 A Product Immediately after Aromatic Ring Cleavage by Lignin Peroxidase

3. 2. 1 Introduction

Since the products of aromatic ring cleavage of lignin substructure models by lignin peroxidase were identified as described in the precedent section, the next problem was to elucidate the mechanism for the aromatic ring cleavage. First of all, tracer experiments with $H_2{}^{18}O$ were conducted, which will be described in Section 3. 3. However, the products described in Section 3. 1 were supposed not to be immediate products after ring cleavage, and the mechanism was not fully elucidated[47]. In the present section, the first identification of an immediate product after ring cleavage is described[78], which made it possible to clarify the aromatic ring cleavage mechanism.

3. 2. 2 Results

α, γ-Diethyl ether of arylglycerol-β-guaiacyl ether (I-Et) (3 μmol) was incubated

with lignin peroxidase for 30 min at 37°C under air as described in the previous section. The degradation products were extracted and the extract was analyzed by GC-MS. GC-MS analysis of the degradation products showed the formation of a novel aromatic ring cleavage product, methyl muconate of 1, 3-diethoxy-1-(4-ethoxy-3-methoxyphenyl)-2-propanol (XVIII-Et). The mass spectrum [m/z (%): 436 (M^+, 1.3), 210(16.5), 209(100), 181(13.7), 153(7.0), 151(15.5), 149(8.5), 125(7.7), 111(5.8), 93(10.8)] and the retention time on GC of the product were identical to those of synthesized authentic sample, methyl *cis, cis*-muconate of arylglycerol-α, γ-diethyl ether (XVIII-Et) (Fig. 26). The identification of the product was further confirmed by its derivatization to methyl adipate of 1, 3-diethoxy-1-(4-ethoxy-3-methoxyphenyl)-2-propanol (XIX-Et) by catalytic reduction (10% palladium on carbon/H_2 in CH_3OH) after alumina TLC separation. The mass spectrum [m/z (%): 440(M^+, 0.9), 210(13.6), 209(100), 181(10.9), 165(11.2), 153(5.1), 151(7.7), 149(11.4), 137(10.8), 125(3.2), 111(7.5), 93(8.5)] and the retention time on GC of the reduction product were identical to those of synthesized authentic sample (XIX-Et). The geometrical configurations of the enzymatic reaction product, methyl muconate of arylglycerol-α, γ-diethyl ether (XVIII-Et), was tentatively assigned as *cis, cis*-form, since a geometrical isomer of (XVIII-Et) (*trans, trans*- or *cis, trans*-form) might have the same mass spectrum and retention time. Even if the product would not be *cis, cis*-isomer, the immediate product of aromatic ring cleavage of (I-Et) would be

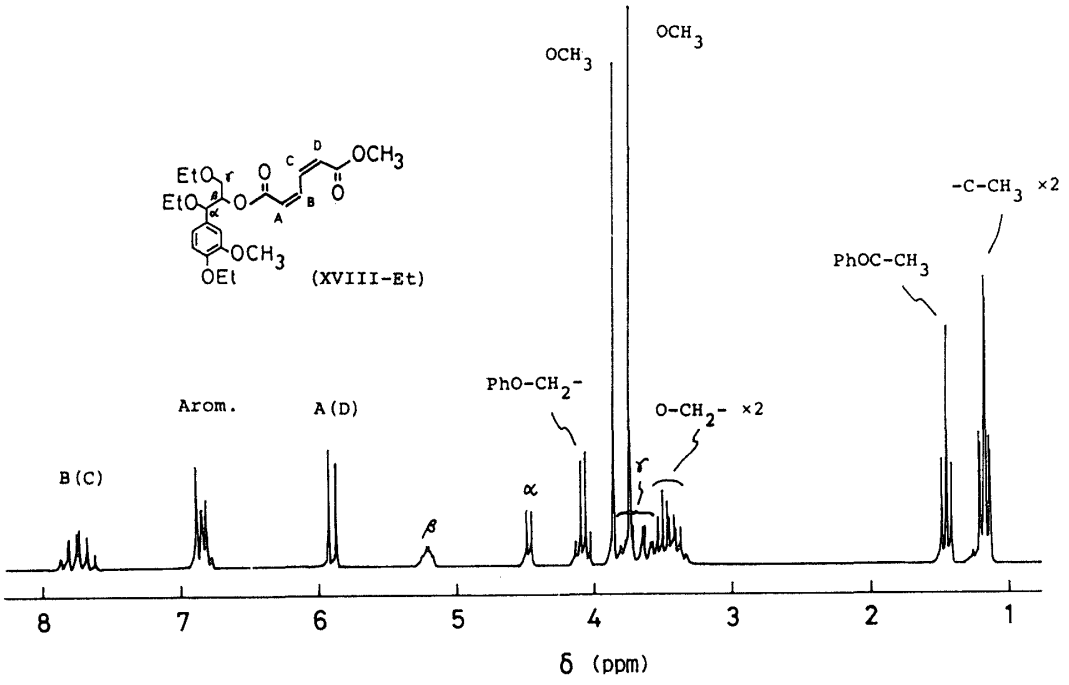

Fig. 26. ^{1}H-NMR spectrum of synthesized methyl *cis, cis*-muconate of 1, 3-diethoxy-1-(4-ethoxy-3-methoxyphenyl)-2-propanol, solvent: $CDCl_3$.

the *cis, cis*-isomer considering its formation mechanism which is discussed in Section 3.3[79].

When α,γ-diethyl ether of arylglycerol-β-[U-*ring*-^{13}C] guaiacyl ether (I-Et-^{13}C) was degraded by the enzyme system, the mass spectrum of the muconate from (I-Et-^{13}C) [molecular ion region of the mass spectrum, m/z (%): 443(24.5), 442(100), 441 (40.6), 440(17.8), 439(1.4), 438(0), 437(0), 436(0)] on GC-MS analysis exhibited a molecular ion peak higher by 6 mass units than that of unlabeled authentic sample (M^+, m/z 436). The result confirmed that methyl muconate of arylglycerol-α,γ-diethyl ether (XVIII-Et) is the product of B-ring cleavage of (I-Et).

Based on a stable isotope dilution method, the yield of (XVIII-Et) was 0.5 μg when large excess of α,γ-diethyl ether of [OCD_3] arylglycerol-β-guaiacyl ether (I-Et-D) (122 μg, 0.3 μmol) was oxidized by the enzyme/H_2O_2 system, where substrate (I-Et-D) still remained after incubation. Neither denatured enzyme (boiled at 100°C for 5 min)/H_2O_2 system nor the system without H_2O_2 catalyzed the formation of (XVIII-Et) from α,γ-diethyl ether of arylglycerol-β-guaiacyl ether (I-Et).

3. 2. 3 Discussion

Our previous investigations demonstrated that aromatic ring cleavage reactions of β-O-4 lignin substructure models were mediated by intact cultures of *Phanerochaete chrysosporium* (Chapter 2)[68,70] and *Coriolus versicolor*[71], and also by lignin peroxidase of *P. chrysosporium* (Section 3. 1)[46,47]. There, four esters of arylglycerols were found as aromatic ring cleavage products (two cyclic carbonates, formate and methyl oxalate of arylglycerols). Subsequently, Miki *et al.* also identified three aromatic ring cleavage products including cyclic carbonates in degradation of another β-O-4 model by the enzyme[80]. However, these compounds would not be suitable for clarification of the mechanisms of aromatic ring cleavage, since they does not retain total carbon atoms of B-ring of the substrates, β-O-4 lignin models.

In the preliminary experiment of degradation of 1-(4-ethoxy-3-methoxyphenyl)-1, 3-diethoxy-2-(2, 6-dimethoxyphenoxy) propane (II-Et) by the enzyme system, another new product was detected, which was eluted from GC column after substrate (II-Et) (data not shown)[47]. The product could not be acetylated by acetylation (Ac_2O/pyridine, room temp.) suggesting that the product has no acetylatable hydroxyl group. Molecular ion of the product seemed to be m/z 466 (base peak m/z 209) which is 32 mass unit higher than that of (II-Et) (m/z 434) suggesting the addition of two oxygen atoms to the substrate. $H_2{}^{18}O$ incorporation experiment showed that one of the oxygen atoms of the product was derived from $H_2{}^{18}O$. These results suggested that one of the possible structures of the product is a methyl methoxymuconate of 1, 3-diethoxy-1-(4-ethoxy-3-methoxyphenyl)-2-propanol, probably an immediate aromatic ring cleavage product[47]. Subsequently, the present investiga-

tion by the use of another β-O-4 lignin substructure model dimer (I-Et) analogous to (II-Et) demonstrated identification of methyl muconate of 1, 3-diethoxy-1-(4-ethoxy-3-methoxyphenyl)-2-propanol (XVIII-Et) by comparison of its mass spectrum and retention time with those of the authentic sample[78]. This is the first paper on the demonstration of identification of an initial product immediately after aromatic ring cleavage of β-O-4 lignin substructure model dimer (I-Et) by the enzyme, which retains six carbon atoms derived from B-ring of (I-Et) (Fig. 27). Recently, muconate derivatives as ring cleavage products of a monomeric aromatic compound, veratryl alcohol, by the enzyme was reported[75]. Further study of the mechanism of aromatic ring cleavage by lignin peroxidase was conducted by the use of $^{18}O_2$, $H_2{}^{18}O$ and deuterated substrate. The proposed mechanism for formation of methyl muconate of arylglycerol-α, γ-diethyl ether (XVIII-Et) from α, γ-diethyl ether of arylglycerol-β-guaiacyl ether (I-Et) will be described in Section 3. 3[79].

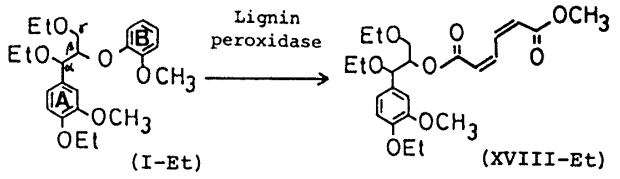

Fig. 27. Methyl muconate of arylglycerol-α, γ-diethyl ether (XVIII-Et), an immediate product of aromatic ring cleavage of β-O-4 lignin model dimer (I-Et) by lignin peroxidase.

3. 3 Mechanism for Aromatic Ring Cleavage by Lignin Peroxidase

3. 3. 1 Introduction

In the precedent section, identification of the immediate product after aromatic ring cleavage of a β-O-4 lignin substructure model by lignin peroxidase was described[78]. One of the next problems to be solved was to elucidate the ring cleavage mechanisms. Based on the results of the experiments using $H_2{}^{18}O$, $^{18}O_2$ and deuterium, mechanisms for aromatic ring cleavage by lignin peroxidase were proposed[79].

3. 3. 2 Results

β-O-4 lignin substructure model dimers etherified at the Cα (and Cγ) positions, (II-Et) and (II-Me), were used in ^{18}O-incorporation experiments, since some of products from these substrates can be submitted directly to GC-MS analysis without acetylation to minimize possible decrease in ^{18}O content by exchange with moisture ($H_2{}^{16}O$). In a preliminary experiment, α-methyl ether of arylglycerol-β-(2, 6-dimethoxyphenyl) ether (II-Me) which represents β-O-4 substructures etherified at α position (substructures 2–3, 8–9, 24–27 in Fig. 1) was incubated with the isotopically unmodified enzyme system. Arylglycerol-α-methyl ether (XI-Me-Ac) [MS m/z (%): 340(M^+, 2.3), 196(14.3), 195(100), 167(40.4), 152(8.6), 151(8.4)], cyclic carbonate

of arylglycerol-α-methyl ether (XIV-Me) [MS m/z (%): 282(M^+, 5.6), 196(12.1), 195(100), 167(61.0), 152(14.7), 151(9.4)], formate of arylglycerol-α-methyl ether (XVI-Me-Ac) [MS m/z (%): 326(M^+, 2.8), 196(11.7), 195(100), 167(41.3), 152 (8.4), 151(6.3)] and methyl oxalate of arylglycerol-α-methyl ether (XVII-Me-Ac) [MS m/z (%): 384(M^+, 3.7), 196(12.5), 195(100), 167(33.2), 152(7.7), 151(6.3)] were identified as products by GC-MS analysis after acetylation. The spectrum and retention time of (XI-Me-Ac) were identical to those of synthesized authentic compound. Formation of methyl oxalate of arylglycerol-α,γ-diethyl ether (XVII-Et) in the degradation of (II-Et) by the enzyme system was reported in Section 3. 1. Furthermore, arylglycerol-α,γ-diethyl ether (XI-Et-Ac) [MS m/z (%): 340(M^+, 2.0), 209(100), 181(16.0), 153(8.1), 151(4.3), 125(8.8), 93(11.0)] was identified as another product by comparison of the mass spectrum and the retention time with those of authentic sample.

β-O-4 lignin substructure model dimers, (I-Et), (II), (II-Et) and (II-Me) were degraded by lignin peroxidase under $H_2{}^{18}O$ or $^{18}O_2$. The degradation products were extracted with ethyl acetate, and the extracts were analyzed by GC-MS immediately after evaporation of ethyl acetate or after acetylation (acetic anhydride/pyridine=1:1, by vol., 10 hr, room temp.). Results of incorporation of ^{18}O from

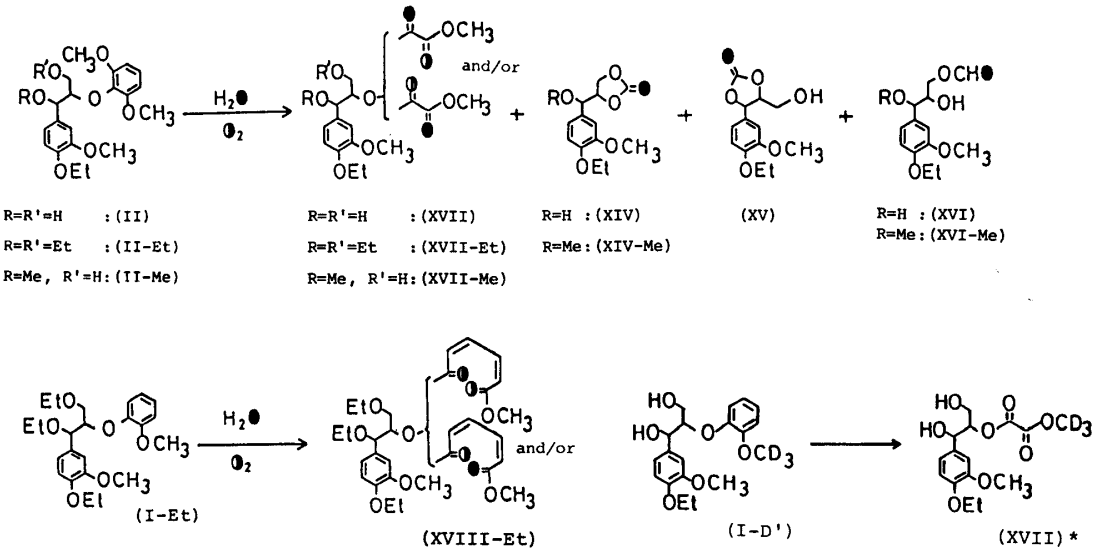

Fig. 28. ^{18}O incorporation from $^{18}O_2$ and $H_2{}^{18}O$ into aromatic ring cleavage products in the degradation of β-O-4 lignin model dimers by lignin peroxidase, and no demeth(ox)ylation during aromatic ring cleavage by the enzyme. ●: ^{18}O of $H_2{}^{18}O$, ◑: ^{18}O of $^{18}O_2$, D: ^2H. Positions of oxalyl groups of (XVII) and (XVII-Me), and formyl group of (XVI-Me) were assigned tentatively at the β- and γ-positions, respectively based on formation of (XVII-Et) from (II-Et), and (XVI) from (II). (XVII)*: [$^2H_3$] methyl oxalate of 1-(4-ethoxy-3-methoxyphenyl)-1, 2, 3-propanetriol.

Table 5. Relative intensities of molecular ion region of mass spectra of products formed on enzymatic oxidation of β-O-4 lignin models under $H_2{}^{18}O$ or $^{18}O_2$.

5-a Muconate (XVIII-Et) formed from (I-Et)

m/z	Relative intensities (%)				
	$H_2{}^{18}O$[a]	$^{18}O_2 \rightarrow H_2{}^{16}O$[b]	$^{18}H_2O$[c]	$^{18}O_2 \rightarrow H_2{}^{16}O$[d]	authentic[e]
434	0	0	4.9	4.9	0
436[g]	91.4	89.1	15.2	12.2	100
438	100	100	100	100	5.0
440	5.2	1.8	12.3	23.2	0

5-b Oxalate (XVII-Et) formed from (II-Et)

m/z	Relative intensities (%)				
	$H_2{}^{18}O$		$^{18}O_2$	$^{18}O_2 \rightarrow H_2{}^{16}O$	authentic
382	0	(0)[f]	0	0	0
384[g]	100	(100)	20.7	36.4	100
386	80	(80)	100	100	3.7
388	0	(0)	21.4	27.1	0

5-c Oxalate (XVII) formed from (II)

m/z	Relative intensities (%)		
	$^{18}O_2$	$^{18}O_2 \rightarrow H_2{}^{16}O$	authentic
410	0	0	0
412[g]	11.8	11.1	100
414	100	100	6.5
416	5.1	5.3	0

5-d Oxalate (XVII-Me) formed from (II-Me)

m/z	Relative intensities (%)				
	$H_2{}^{18}O$	$H_2{}^{18}O \rightarrow H_2{}^{16}O$	$^{18}O_2$	$^{18}O_2 \rightarrow H_2{}^{16}O$	authentic
382	0	0	0	0	0
384[g]	100	100	36.6	32.3	100
386	97.1	86.8	100	100	7.2
388	17.5	0	7.6	0	0

5-e Cyclic carbonate (XIV) formed from (II)

m/z	Relative intensities (%)		
	$^{18}O_2$	$^{18}O_2 \rightarrow H_2{}^{16}O$	authentic
308	0	0	0
310[g]	100	100	100
312	4.0	3.9	2.6

Table 5. (cont'd)

5-f Cyclic carbonate (XV) formed from (II)

m/z	Relative intensities (%)		
	$^{18}O_2$	$^{18}O_2 \rightarrow H_2{}^{16}O$	authentic
308	0	0	0
310[g]	100	100	100
312	6.2	7.3	2.9

5-g Cyclic carbonate (XIV-Me) formed from (II-Me)

m/z	Relative intensities (%)									
	$H_2{}^{18}O$		$H_2{}^{18}O \rightarrow H_2{}^{16}O$		$^{18}O_2$		$^{18}O_2 \rightarrow H_2{}^{16}O$		authentic
280	0	(0)[f]	0	(0)[f]	9.7	(1.4)[f]	11.9	(0)[f]	0
282[g]	100	(100)	100	(100)	100	(100)	100	(100)	100
284	89.2	(90.9)	93.8	(92.5)	4.6	(3.0)	6.0	(4.2)	2.6

5-h Formate (XVI) formed from (II)

m/z	Relative intensities (%)		
	$^{18}O_2$	$^{18}O_2 \rightarrow H_2{}^{16}O$	authentic
352	0	0	0
354[g]	100	100	100
356	3.5	2.8	2.9

5-i Formate (XVI-Me) formed from (II-Me)

m/z	Relative intensities (%)				
	$H_2{}^{18}O$	$H_2{}^{18}O \rightarrow H_2{}^{16}O$	$^{18}O_2$	$^{18}O_2 \rightarrow H_2{}^{16}O$	authentic
324	0	0	0	0	0
326[g]	100	100	100	100	100
328	68.2	34.0	2.7	2.4	4.5

a), b): Incubation product under $H_2{}^{18}O$ and the product of its re-incubation under $H_2{}^{16}O$, respectively (^{18}O content in $H_2{}^{18}O$ of the medium: 49 atom%). c), d): Incubation product under $^{18}O_2$ (^{18}O: 98.58%) and the product of its re-incubation under $H_2{}^{16}O$, respectively. e): Unlabeled authentic sample. f): Analyzed after acetylation. g): Molecular ion of unlabeled authentic sample. (XVII), (XVII-Me), (XIV), (XV), (XVI) and (XVI-Me): Analyzed after acetylation.

$H_2{}^{18}O$ and $^{18}O_2$ are shown in Table 5 which is summarized as follows (Fig. 28).

In the formation of methyl muconate of arylglycerol-α,γ-diethyl ether (XVIII-Et) from α,γ-diethyl ether of arylglycerol-β-guaiacyl ether (I-Et), one atom of ^{18}O was incorporated into the carbonyl groups of the muconate (XVIII-Et) from $H_2{}^{18}O$, and another atom of ^{18}O from $^{18}O_2$.

One atom of ^{18}O was incorporated into the carbonyl groups of methyl oxalates

of arylglycerols (XVII-Et), (XVII) and (XVII-Me) from $^{18}O_2$ in the degradation of arylglycerol-β-(2, 6-dimethoxyphenyl) ether (II), α-methyl ether of arylglycerol-β-(2, 6-dimethoxyphenyl) ether (II-Me) and α, γ-diethyl ether of arylglycerol-β-(2, 6-dimethoxyphenyl) ether (II-Et); another atom of ^{18}O from $H_2{}^{18}O$. (Incubation of (II) under $H_2{}^{18}O$ was not made).

^{18}O incorporation into cyclic carbonates of arylglycerols (XIV), (XV) and (XIV-Me) was not found in the incubation of arylglycerol-β-(2, 6-dimethoxyphenyl) ether (II) and α-methyl ether of arylglycerol-β-(2, 6-dimethoxyphenyl) ether (II-Me) under $^{18}O_2$ by the enzyme. On the other hand, one atom of ^{18}O was incorporated into the carbonyl oxygen of (XIV-Me) in the incubation of (II-Me) under $H_2{}^{18}O$.

In the incubation of arylglycerol-β-(2, 6-dimethoxyphenyl) ether (II) and α-methyl ether of arylglycerol-β-(2, 6-dimethoxyphenyl) ether (II-Me) under $^{18}O_2$ by the enzyme, ^{18}O incorporation into the formates (XVI) and (XVI-Me) was not found. On the other hand, one atom of ^{18}O was incorporated into the carbonyl oxygen of (XVI-Me) in the incubation of (II-Me) under $H_2{}^{18}O$.

The ^{18}O contents of the products except for formate of arylglycerol-α-methyl ether (XVI-Me) were not reduced on re-incubation of the ^{18}O-incorporated products with $H_2{}^{16}O$ medium, while that of (XVI-Me) was reduced partly (Table 5-i).

Arylglycerol-β-[OCD_3] guaiacyl ether (I-D'), which has a deuterated methoxyl group on B-ring, was used as a substrate and degraded by the lignin peroxidase system. GC-MS analysis (mass chromatography) showed that the molecular ion peak of (XVII-Ac) derived from (I-D') was m/z 415, 3 mass units higher than that from

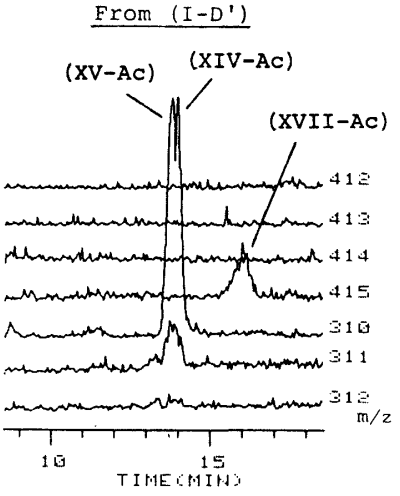

Fig. 29. Mass chromatogram of molecular ion peaks of degradation products from (I-D'). Molecular ion of (XVII-Ac) is m/z 415, three mass units higher than that of unlabeled authentic sample (m/z 412, Fig. 25, A).

isotopically unmodified analog (II) (Fig. 29). Accordingly, methyl group of methyl ester of the oxalate was evidenced to be derived from methoxyl group on B-ring of (I-D').

3. 3. 3 Discussion

Based on the results of tracer experiments by the use of $H_2{}^{18}O$, $^{18}O_2$ and deuterium[47,79], the following mechanisms were proposed for aromatic ring cleavage of β-O-4 lignin substructure model dimers by lignin peroxidase/H_2O_2 system to give muconate, oxalates and cyclic carbonates of arylglycerols (Fig. 30)[79]. Lignin peroxidase is known to produce cation radicals from methoxylated benzenes including lignin substructure model dimers[46,45]. The mechanisms involve single-electron oxidation of the aromatic ring to the corresponding cation radical [substrate→(a)], followed by attack by a nucleophile (H_2O or the hydroxyl groups of Cα and Cγ positions of the propyl side chain) [(a)→(b), (a)→(g)], and coupling with O_2 [(b)→(c), (e)→(f), (g)→(h)]. Intramolecular addition of peroxy radicals to double bonds to form cyclic peroxides and subsequent coupling with O_2 were demonstrated by Porter *et al.*[81]. The mechanism for aromatic ring cleavage by lignin peroxidase proposed here is in accord with the experimental results obtained (Fig. 28). Formation of α,β-cyclic carbonate (XV) is explained as in the formation of β,γ-cyclic carbonate (XIV) (Fig. 30): Intramolecular nucleophilic attack by Cα-hydroxyl function of (a) in Fig. 30 to produce the positional isomer of (g) in Fig. 30 [the same as (d) in Fig. 15A] followed by its decomposition to give (XV) *via* a pathway similar to (g)→(XIV) in Fig. 30. Recent study showed that ring cleavage of a β-O-4 lignin model by the enzyme occurred only in the presence of O_2 which is in accord with the mechanisms shown in Fig. 30, and a radical analogous to (g) (Fig. 30) was suggested for the formation of cyclic carbonates analogous to (XIV) and (XV)[80]. In the present investigation, the mechanisms involving nucleophilic attack by H_2O to the cation radicals were proposed for the first time for the aromatic ring cleavage by the enzyme.

Instead of O_2, other radicals derived from O_2 might be involved[79,82] (*e.g.* the coupling of (b) with hydroperoxy radical, which was suggested to be formed from O_2 during oxidation of lignin model compounds by the enzyme[66,83]). Since the present result showed the incorporation of ^{18}O from $H_2{}^{18}O$ into the muconate and the oxalates of arylglycerols, the coupling of the cation radical (a) with hydroperoxy radical to give a dioxetane was not essential in their formation, which was assumed recently[83]. The formation mechanism of formates of arylglycerols (XVI) and (XVI-Me) was not fully examined in the present investigation. However, the formyl hydrogen atom of (XVI) was found to be derived quantitatively from H_2O by tracer experiment using ^2H_2O (data not shown). Leisola *et al.*[75] reported muconate de-

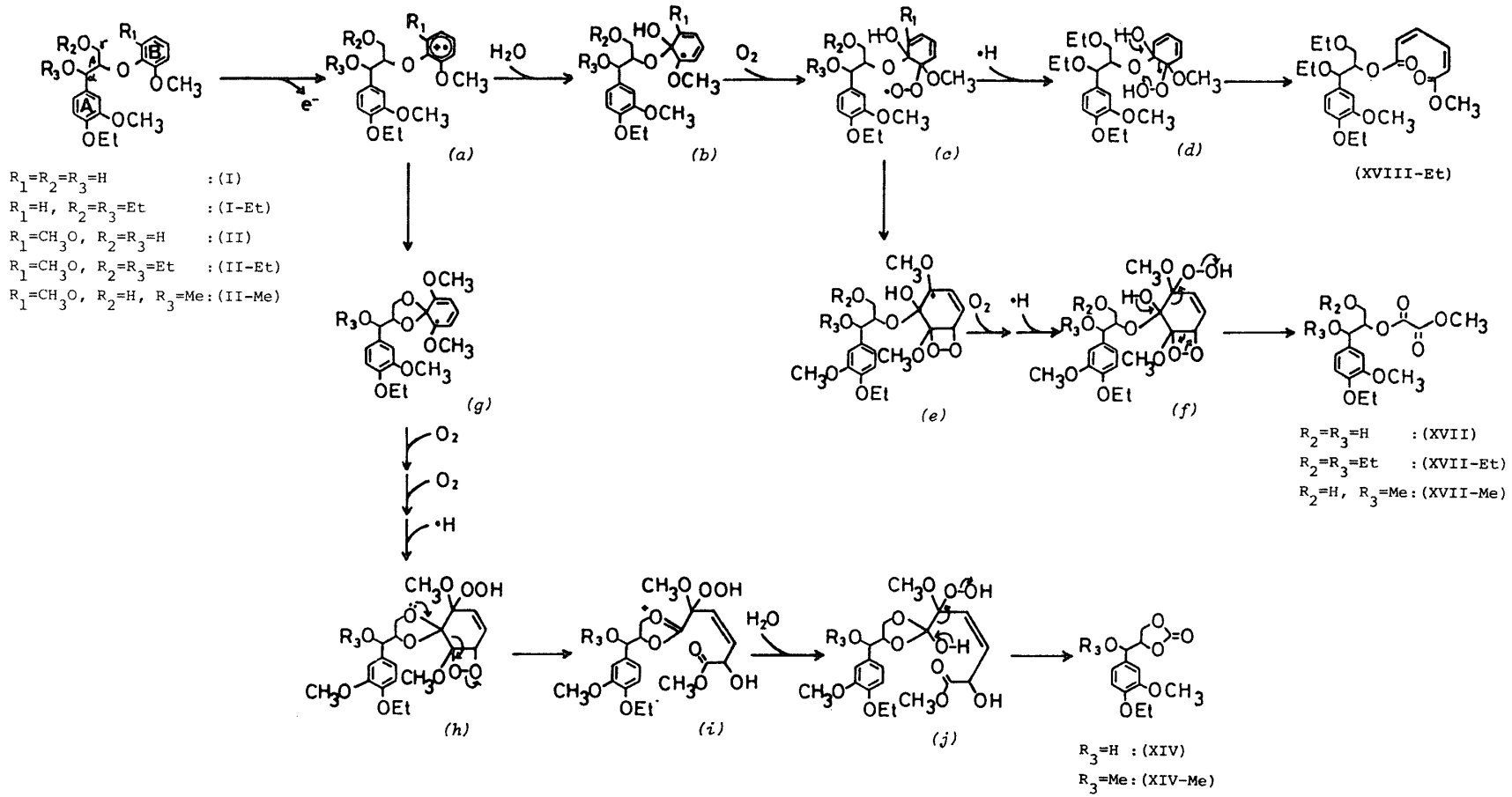

Fig. 30. Proposed mechanisms for aromatic ring cleavage of β-O-4 lignin substructure models by lignin peroxidase. (XVIII-Et) was formed from (I-Et), (XVII) from (I) and (II), (XVII-Et) from (II-Et), (XVII-Me) from (II-Me), (XIV) from (I) and (II), (XIV-Me) from (II-Me). Et: CH_2CH_3, Me: CH_3.

rivatives as ring cleavage products of veratryl alcohol by the enzyme. The mechanisms similar to the formation of muconate (XVIII-Et) and the formation of cyclic carbonates (XIV) and (XV) were also proposed for the lignin peroxidase catalyzed ring cleavage of veratryl alcohol[84], and of a β-O-4 lignin substructure model[85], respectively.

Present result of retention of methoxyl group on B-ring of (I-D′) in methyl oxalate of arylglycerol (Fig. 28) evidenced that demeth(ox)ylation is not prerequisite for the aromatic ring cleavage by lignin peroxidase, while earlier works[69] suggested that demethylation of lignin related methoxylated monomeric aromatic compounds to give *o*-diphenols is prerequisite for ring cleavage by dioxygenases. The mechanism for aromatic ring cleavage by lignin peroxidase is completely different from that for conventional ring cleavage of aromatic compounds catalyzed by dioxygenases. Thus, the aromatic ring cleavage of lignin substructure models by *P. chrysosporium* is different from that of simple monomeric phenols by bacteria and fungi which need conventional dioxygenases, although possible involvement of dioxygenases in the aromatic ring cleavage of lignin-derived monomeric aromatic compounds by *P. chrysosporium* is still open. Analysis of decayed lignin isolated from decayed wood showed formation of benzoic acids and benzaldehydes such as vanillic acid, syringic acid, vanillin and syringaldehyde during decomposition of lignin by white-rot fungi. These benzoic acids and benzaldehydes might be metabolized *via* decarboxylation, demethylation, subsequent ring opening by dioxygenases and conventional β-keto adipate pathway by *P. chrysosporium* as proposed by Ander *et al.*[86].

Besides cleavage of aromatic ring and cleavage of Cα-Cβ and β-O-4 bond, several reactions were found in the degradation of β-O-4 lignin substructure models by cultures of *P. chrysosporium* and by lignin peroxidase of the fungus. Among them are found Cα oxidation and formation of cyclohexadienone ketals. Mechanisms proposed for the reactions were also based on initial cation radical formation by the enzyme[45,87]. Thus, most of the degradative reactions of β-O-4 lignin substructure models mediated by cultures of *P. chrysosporium* are explained on the basis of single-electron oxidation by the enzyme [Figs. 14, 15, 30]. This mode of degradation is non-specific, producing a variety of products depending on the nature of the substrates. It is regarded as an autoxidative degradation rather than a series of highly specific enzymatic reactions involving physiological metabolic pathways. Lignin peroxidase was shown to be one of the hemoproteins[38,42]. Degradative reactions of lignin substructure model compounds by a heme-enzyme model catalyst[88–91] and by single-electron transfer reagents[67,92] were reported. For the "biomimetic" reactions, mechanisms involving single-electron oxidation and cation radical formation were proposed.

The mechanisms for the degradation of lignin substructure models by the enzyme

proposed in this work (Figs. 15 and 30) are based on the studies using β-O-4 lignin substructure model dimers which have no propyl side chains on their B-rings. However, the mechanisms are probably the case with degradative reactions of lignin macromolecules by the enzyme at least qualitatively, because recently the same products of aromatic ring cleavage and β-O-4 bond cleavage as in the degradation of β-O-4 dimers by the enzyme were identified in the degradation of a lignin model trimer (XXX) which has a propyl side chain on the B-nucleus by the enzyme[93], and of a special DHP (dehydrogenation polymer, synthetic lignin) composed of arylglycerol-β-syringaresinol ether (XXXI) and coniferyl alcohol (XXXII) by the enzyme (Fig. 31)[94]. Furthermore, earlier study showed the same Cα-Cβ cleavage products as in the degradation of lignin substructure model dimers were formed in degradation of spruce and birch lignins by the enzyme[36].

Lignin peroxidase is not only produced by *P. chrysosporium*, but also produced by other fungi, since similar enzymes are beginning to be characterized[95–98]. It is, therefore, probable that initial stages of lignin degradation by the fungi producing

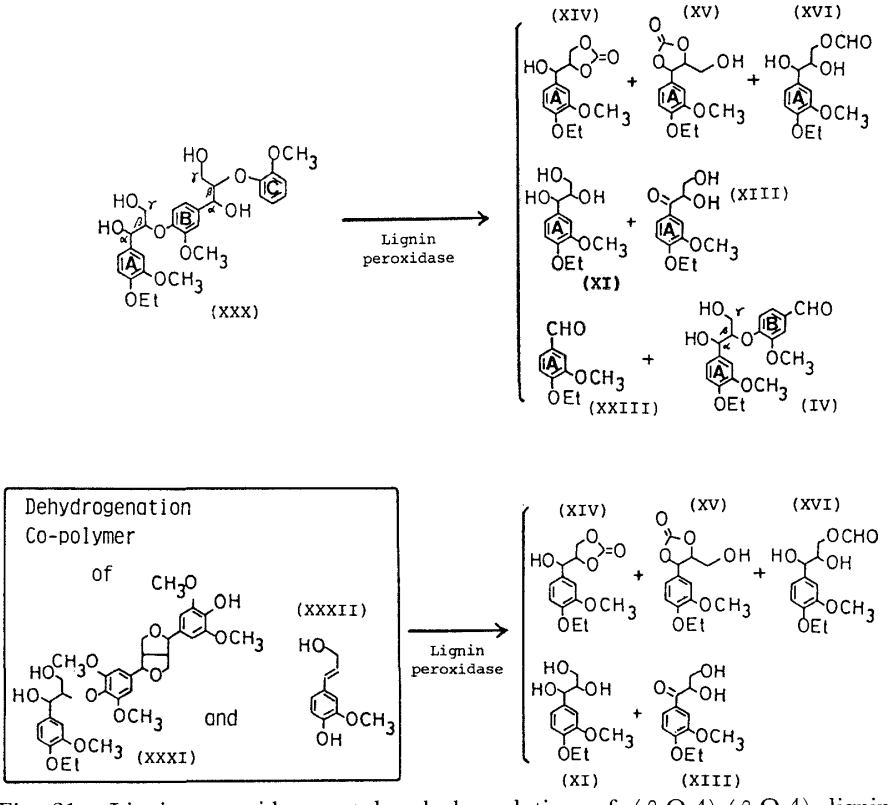

Fig. 31. Lignin peroxidase catalyzed degradation of (β-O-4)-(β-O-4) lignin substructure model trimer (XXX) and DHP composed of arylglycerol-β-syringaresinol ether (XXXI) and coniferyl alcohol (XXXII). (XIV), (XV) and (XVI): Aromatic ring cleavage products; (XI) and (XIII): β-O-4 bond cleavage products; (XXIII) and (IV): Cα-Cβ cleavage products.

lignin peroxidase are an autoxidative process initiated by single-electron oxidation catalyzed by the enzyme.

Conclusion

Mechanism of lignin biodegradation has been investigated by two complementary approaches: (i) Fungal degradation of lignin polymer and (ii) fungal degradation of lignin substructure models[3,4]. Until early 1980s, analysis of decayed lignin isolated from decayed wood by white-rot fungi showed that at least three modes of degradative reactions occur in the decomposition of lignin by the fungi: (i) cleavage between Cα and Cβ of the propyl side chain, (ii) cleavage of β-O-4 ether bond, and (iii) aromatic ring cleavage[4]. While in degradation studies of lignin substructure models, in early 1980s, products of Cα-Cβ cleavage and β-O-4 bond cleavage were identified based on mass spectrometric analysis[14,15,55]. And two hypotheses were proposed for the β-O-4 bond cleavage: Cβ-hydroxylation and hydrolysis of β-O-4 bond. However, no concrete evidences were provided supporting the speculated mechanisms for the reactions. Furthermore, products of aromatic ring cleavage were not identified.

The object of the present investigation is to elucidate the cleavage mechanisms of side chain and aromatic ring.

First, the author investigated β-O-4 and α-O-γ type lignin substructure model trimer with ligninolytic culture of *Phanerochaete chrysosporium* (Section 1. 1), showing that two alternative pathways were involved in the degradation of the β-O-4 lignin model[20,21]. One was *via* arylglycerol, and the other oxygenative Cα-Cβ cleavage.

Second, mechanisms for β-O-4 bond cleavage to produce the arylglycerol by *P. chrysosporium* was studied. Tracer experiments by the use of $H_2{}^{18}O$ and deuterated or ^{18}O-labeled substrates evidenced that the two mechanisms proposed by earlier investigators, initial hydroxylation at Cβ position of the β-O-4 lignin model, and direct hydrolysis of the β-O-4 ether bond, are not involved in the arylglycerol formation (Sections 1. 2 and 1. 3)[20,26,31].

In the next section, a new mechanism was proposed for arylglycerol formation from β-O-4 substructure models (Section 1. 4): The arylglycerol was formed *via* cleavage between β-ethereal oxygen and C_4 of the B-nucleus (O-C_4 cleavage)[34].

In Section 1. 5, mechanism for the formation of another product, 2-guaiacoxyethanol, was proposed. This reaction was explained based on initial single-electron oxidation of B-ring to form the corresponding cation radical by the extracellular enzyme of *P. chrysosporium*, lignin peroxidase[39]. Furthermore, of great importance was that all the results of tracer experiments on the β-O-4 bond cleavage to give the arylglycerol formation described in Section 1. 2 to Section 1. 4 was reasonably ex-

plained by cation radical intermediate.

The other problem, aromatic ring cleavage, was investigated in the latter half of this work. In Section 2. 1, first identification of the product of aromatic ring cleavage was described. β,γ-Cyclic carbonate of arylglycerol was produced in cleavage of guaiacyl aromatic ring of arylglycerol-β-guaiacyl ether by intact cells of *P. chrysosporium*[68]. Several other products of aromatic ring cleavage by the fungus were subsequently identified (Section 2. 2)[70].

It has been known that dioxygenases such as protocatechuate 3, 4- or 4, 5-dioxygenase and catechol 1, 2- or 2, 3-dioxygenase are responsible for aromatic ring cleavage of simple aromatic compounds by microorganisms. Aromatic rings of lignin substructure models, however, were found to be cleaved by the extracellular enzyme of *Phanerochaete chrysosporium,* lignin peroxidase[46,47]. Formation of the ring cleavage products described in Chapter 2 was also mediated by lignin peroxidase (Section 3. 1). These products, however, did not keep all the six carbon atoms derived from the B-ring of the substrates, and, therefore, the mechanism for the ring cleavage could not be fully elucidated. In Section 3. 2, an immediate product of aromatic ring cleavage of lignin substructure models by lignin peroxidase, methyl muconate of arylglycerol, was first identified[78]. The product retained all the carbon atoms of the B-ring, and made it possible to clarify the aromatic ring cleavage mechanism.

In Section 3. 3, ^{18}O incorporation into the aromatic ring cleavage products from $H_2{}^{18}O$ and $^{18}O_2$ and degradation of the deuterated substrate were investigated, and the mechanism for aromatic ring cleavage by lignin peroxidase was proposed[79]. The mechanism involves single-electron oxidation of the aromatic ring to the corresponding cation radical, followed by attack by nucleophiles (H_2O and intramolecular hydroxyl groups) and coupling with O_2.

Thus, most of the initial degradative reactions of β-O-4 lignin substructure models mediated by cultures of the fungus were explained on the basis of single-electron oxidation of lignin peroxidase. The mode of degradation is non-specific, and is regarded as an autoxidative degradation rather than a series of highly specific enzymatic reactions involving physiological metabolic pathways. The mechanisms for the degradation of lignin substructure models by the enzyme proposed in this work are probably the case with degradative reactions of lignin macromolecules by the enzyme, because recently the same products of aromatic ring cleavage and β-O-4 bond cleavage as in the degradation of β-O-4 dimers by the enzyme were identified in the degradation of DHP (synthetic lignin) by the enzyme[94]. Accordingly, initial stage of the lignin degradation by the fungi producing lignin peroxidase is probably an autoxidative process initiated by single-electron oxidation catalyzed by the enzyme.

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